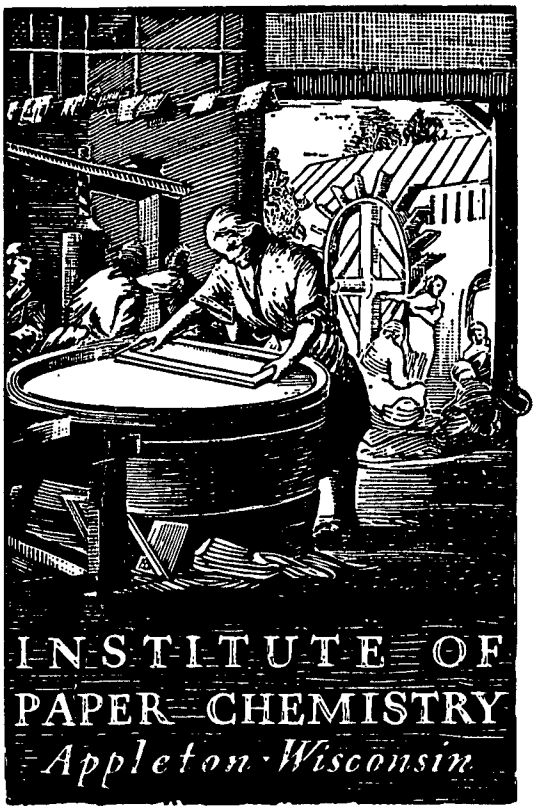

THE MASS PRODUCTION OF CONIFER HYBRIDS

Project 3223

Report Eleven
A Progress Report
to
MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

February 29, 1984

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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SUMMARY

MODEL SYSTEMS RESEARCH

During the past year, model systems research was an important part of the overall tissue culture research effort. Studies on the polyamine levels in wild carrot and in developing pine cone embryos represent just a part of our research effort in this area. As a follow-up to earlier reported metabolic pathway/inhibitor research, a study was established in which the role of arginine decarboxylase (ADC) and polyamines in wild carrot embryogenesis was investigated. The results of these experiments demonstrated that the inhibitor α-difluoromethylarginine (DMFA) caused a 50% reduction in wild carrot embryo formation. Additions of putrescine restored embryogenesis. The inhibition of development by DFMA, which acts by decreasing ADC activity and lowering putrescine and spermidine levels in the cells, demonstrates that putrescine and spermidine are among factors essential for embryogenesis in wild carrot. Additional inhibitor studies which employed dicyclohexylammonium sulfate (DCHA) and methyl glyoxal bis-guanyl hydrazone (MGBG) seem to indicate that spermidine, rather than putrescine, may be the polyamine most required in wild carrot embryo development.

Studies conducted on polyamine levels in pine ovules collected from developing cones demonstrated that polyamines rose at times of suspected fertilization and embryo development. Spermidine levels rose more than either putrescine or spermine, providing an additional example of the similarity between wild carrot and pine embryogenesis.

As part of our research on testing the use of "undefined media" to stimulate embryogenesis, extracts of developing pine seeds were prepared and first tested to determine their influence on wild carrot embryogenesis. Certain fractions are now undergoing similar tests with conifer cell lines. Preliminary results indicate that a high molecular weight fraction has been isolated that promotes wild carrot embryogenesis. The low molecular weight fraction used appears to have a toxic influence on embryogenesis, and its future use will require removal of that part of the low molecular weight fraction causing the toxicity.

Two additional model system studies were established to check on the ability of the new LM to initiate competent cell lines and to determine if there was anything inherent in the makeup of LM that prevented embryogenesis in competent cell lines. The wild carrot model system was used, and the results demonstrated that LM could be used to initiate and maintain competent cell lines and that upon the removal of 2,4-D, satisfactory embryogenesis occurred.

Concern over possible changes that may occur during transport and storage of pine cones resulted in the establishment of two studies, one to examine the effect of cone storage on biochemical parameters and a second to examine the effect of storage on the organogenesis of explants. These two studies demonstrated that free amino acid levels generally increased during storage. Putrescine levels, which are considered sensitive to pH and osmotic stress in plant tissue, also increased quite dramatically with storage. Four-day storage, however, had no influence on immature embryo explants as a source of tissue for organogenic studies.

Growth regulators, particularly IAA, are important in the growth and differentiation of plant structures, and we need to be able to accurately measure growth regulator levels in order to have an appropriate understanding of their importance in embryogenesis. Despite the limitations of current measurement techniques, there appears to be an early increase in IAA content (day 12-14) in both proliferating and organizing wild carrot cells and a second IAA increase during a more advanced stage of wild carrot embryo development (at approximately 3 weeks). Only preliminary data are available on IAA levels in loblolly pine cell suspensions.

OBJECTIVE I RESEARCH

Objective I research, which centers on initiating and maintaining good quality cell suspensions, also received a major amount of emphasis during this past year. One media study investigated phosphate uptake of wild carrot under "plus" growth regulator (proliferative) and "minus" growth regulator (embryogenesis) growth conditions. Our standard 10-D Cotyledon loblolly pine line was also evaluated under similar growth regulator conditions. Wild carrot cultures were found to either (1) remove phosphate prior to growth or (2) remove phosphate very rapidly. As a result the phosphate in the wild carrot medium was depleted by day 9. Higher levels of phosphate in LM delayed depletion until day 14. Loblolly pine cultures, with an inoculum density of 10 µL/mL and "plus" growth regulator, did not deplete the phosphate until day 14. Thus, subculturing 10-D Cotyledon at 10-day intervals is expected to result in adequate cell line phosphate levels (and appropriate energy levels) when tranferred into "minus" growth regulator (embryogenesis) conditions. One possible reason for the slow growth we are experiencing under these conditions could be related to

difficulties in the conversion of phosphate to ATP. A related study, which evaluated lowering calcium and increasing phosphate levels, demonstrated that calcium levels as low as 5.4 ppm (1/4 normal level) and phosphate levels double the normal level could be used in maintaining loblolly pine cell suspensions.

Methods of initiating cell lines from immature embryos were investigated, and a number of cell lines were propagated. No cell lines were obtained from the earliest immature stages of embryo development. Plans have been made for additional studies aimed at improving the efficiency of initiation procedures.

Polyamine levels and enzyme activity in loblolly pine cell suspensions and the influence of polyamine precursors on polyamine levels in loblolly pine cell cultures were two additional investigations undertaken this past year. Earlier reported research demonstrated the importance of polyamines in wild carrot embryogenesis. One of the new studies was designed to determine how polyamines and the enzymes arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) are affected when loblolly pine cell suspensions are subjected to the standard embryogenesis-induction protocol. The results indicate that, although polyamine levels increased at the early sampling times for both the plus and minus 2,4-D cultures, levels were not as high as in the wild carrot cultures. Also, the polyamine putrescine exhibited a significant increase, but quite unexpectedly the activities of the biosynthetic enzymes ADC and ODC did not increase. The second study, which was designed to test for the presence of ADC and ODC inhibitors in loblolly pine cells, failed to locate the inhibitors, and this suggests polyamines may be derived from a source other than (or in addition to) enzymatic biosynthesis.

OBJECTIVE II RESEARCH

Objective II research includes studies designed to induce embryogenesis from cells growing as cell suspensions. Studies undertaken this past year in this phase of our program included a series of unmonitored launch experiments aimed at embryo production, a study of tannin buildup in loblolly pine cell suspensions, and an interesting study in which wild carrot and loblolly pine cells were grown as a mixed cell suspension. This latter study was an attempt to induce loblolly pine embryogenesis by a "co-culture" technique. Results of the co-culture work demonstrated that (1) wild carrot and loblolly pine cells can be grown together and that (2) co-culturing does not inhibit embryogenesis in wild carrot nor does it stimulate embryogenesis in the loblolly pine cells.

The research on tannin in loblolly pine cell suspensions consisted of a series of four experiments designed to (1) determine the relationship between tannin production and the activity of the enzyme phenylalanine ammonia lyase (PAL) and to (2) determine if inhibition of PAL activity is a feasible approach for controlling tannin production. The results of these four experiments demonstrated that (1) there was no strong correlation between PAL activity and tannin production, (2) tannin itself was not solely responsible for the failure of pine cells to grow in a 2,4-D free media and (3) that although the use of PAL inhibitors prevented the appearance of tannins, this did not result in growth response. Of particular interest in this series of studies was the last study in which, by proper selection of the age of the inoculum, some growth occurred and tannin buildup was delayed until about day eight. This suggests that by the proper use of inhibitors along with careful selection of the inoculum age, growth in 2,4-D-free media without tannin buildup may be feasible.

Plans for the coming year include additional model systems research on (1) polyamine levels and associated enzyme activity during the early stages of natural pine embryogenesis, (2) growth regulator changes during natural conifer and wild carrot somatic embryogenesis, (3) the factors important in the conversion of arginine to polyamines, and (4) growth rate and cell division during early stages of embryogenesis. Research on generating and maintaining competent cell lines is expected to include (1) generation of new cell lines from immature embryos, protoplasts, and microsporophyll tissue; (2) determination of the influence of nitrogen sources, polyamines, gibberellins, and cytokinins on conifer cell line quality; (3) determining the influence of natural conifer extracts on conifer cell line quality. Conifer somatic embryogenesis studies are expected to include (1) establishing monitored launch experiments that have as objectives the correction of apparent deficiencies and the production of inhibitors, (2) conducting occasional unmonitored launch experiments incorporating a combination of promising factors, (3) running monitored launch experiments using promising new cell lines, and (4) conducting studies with the objective of determining the influence of natural extracts on conifer embryogenesis.

INTRODUCTION

The Institute's philosophy on solving the embryogenesis problem in conifers is to use a biochemically oriented model systems approach. The recent entry of several organizations into the field of genetic engineering of forest tree species makes existing research groups wonder where we have gone wrong. Their decision to jump directly into genetic engineering without having an appropriate background of producing callus cultures, organogenesis, and cell suspensions, or without having worked on embryogenesis suggests that somehow they know something about tissue culture systems that we do not. They obviously feel that cell line origin, early cell line quality, "competence" and the biochemistry of embryogenesis are mundane problems that everyone understands and are factors that require no additional fundamental research. The Institute's program and overall approach appears to be a direct contradiction of this line of reasoning.

The Institute's model systems research approach was selected in order to make it possible for us to develop a fundamentally sound embryogenesis system. We feel that by knowing the biochemistry of our system and having appropriate biochemical and morphological markers, we can remove tissue culture from the status of an "art" into the realm of a science. We want embryogenesis to be a controlled, not a chance event.

The studies that are described in the report that follows are intended to provide a better understanding of the biochemistry of embryogenesis and to allow us to move toward the development of a fundamentally sound system.

MODEL SYSTEMS RESEARCH

THE ROLE OF ARGININE DECARBOXYLASE AND POLYAMINES IN WILD CARROT EMBRYOGENESIS

Introduction

In our first studies on the metabolism of plant tissues during embryogenesis and development we concentrated on changes in free amino acid pools. Arginine was found to be a dominant amino acid, at times comprising over 50% of the free amino acid pool, and it also exhibited large fluctuations in levels during embryo formation. Arginine appeared to be important in the development/differentiation of both wild carrot and natural pine ovules/seeds. Study of the possible roles of arginine in plant development led logically to experiments on putrescine and the polyamines, compounds which are synthesized from arginine (Fig. 1). Polyamines were also of interest because of their association with growth and developmental processes in other plant, animal and bacterial species (1,2). In our lab, levels of polyamines and the enzymes leading to polyamine biosynthesis were first studied in embryogenic cultures of wild carrot (Daucus carota). Wild carrot was chosen because of the ease of monitoring and manipulating the cells during embryogenesis. The data, reported in Project 3223 Report Ten (1983), demonstrated that rises in ADC (arginine decarboxylase) and polyamines accompany embryogenesis in wild carrot. Peaks of putrescine and ADC were observed three to five days after the induction of embryogenesis.

Determination of the function of polyamines during embryogenesis was made possible by the use of the enzyme inhibitors DFMA (α-difluoromethylarginine) and DFMO (α-difluoromethylornithine), which inhibit the activity of ADC and ODC (ornithine decarboxylase), respectively, (2). We confirmed that these

compounds, designed for use against bacterial enzymes, were capable of inhibiting the enzymes extracted from wild carrot and loblolly pine tissue. The experiments described below were designed to test the effects of these polyamine biosynthesis inhibitors on wild carrot embryogenesis and intracellular polyamine levels.

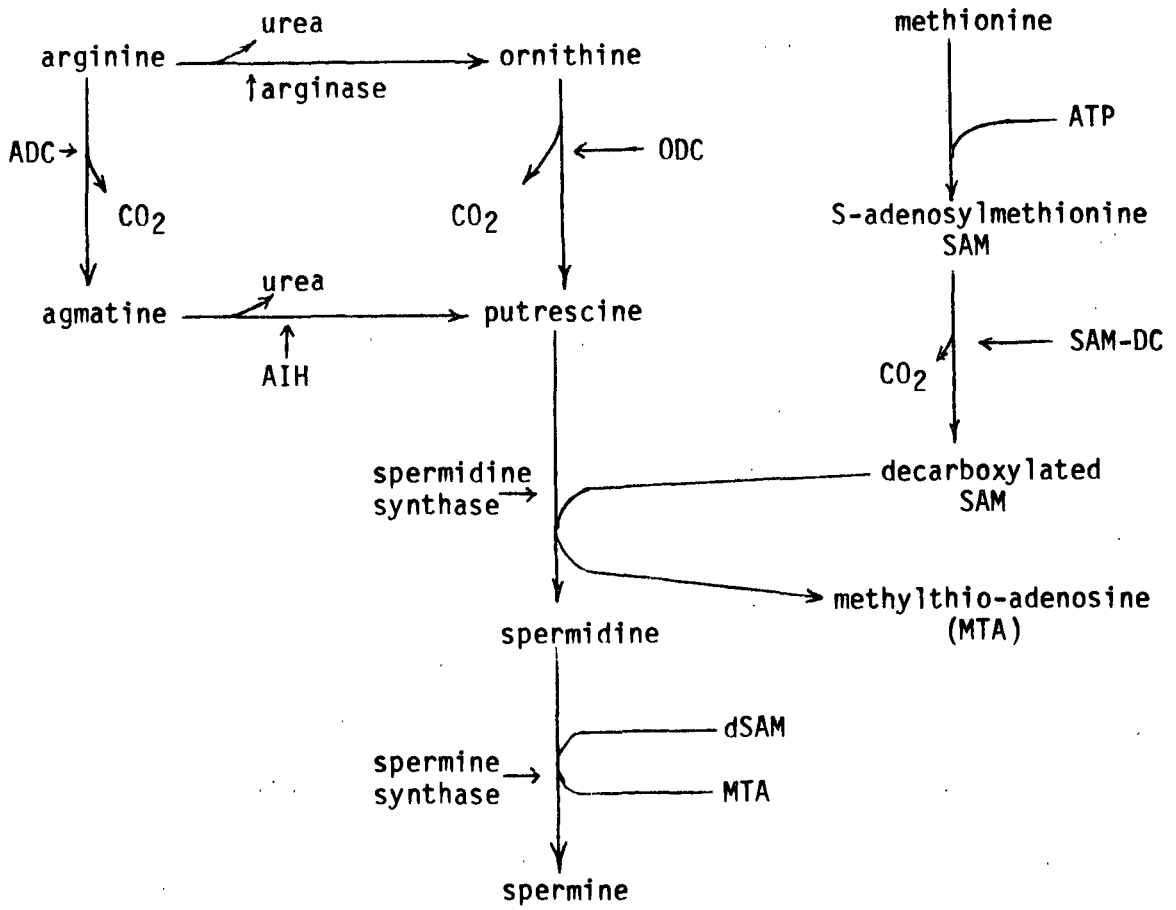


Figure 1. Biosynthesis of polyamines in plants.

Methods

A wild carrot cell line maintained as a suspension culture served as the experimental tissue. Embryo development was induced in the cultures by the standard procedure of screening the suspension to yield cell aggregates of a specific size (63 to 125 μm) and of subculturing these cells into medium lacking 2,4-D (normally present at 0.5 mg/mL). After 23 days of growth in darkness in screw cap roller tubes containing 2 mL medium, a structure was scored as an embryo if it was > 2 mm in length and exhibited both root and shoot development. Embryo count values represent the mean and the standard deviation of at least 6 replicates from a representative experiment. The DFMA and DFMO were added to the cultures at day 0 of culture growth (when cells were screened and subcultured).

To determine the effects of the inhibitors on polyamine levels, polyamines were measured in 5% perchloric acid extracts of cells from 6-day old cultures. We previously determined that polyamine levels in our wild carrot cultures are elevated on day 6 of culture growth (data not shown). Embryogenesis was initiated as described above, except that 250 mL flasks containing 50 mL medium were used in order to yield an adequate amount of tissue for analysis. Benzoylated derivatives of the polyamines were separated and quantitated by the HPLC method described by Flores and Galston (3). The polyamine data represent the mean ± standard deviations of quadruplicate determinations.

Results

DFMA at a concentration of 0.5 mM in the medium did not significantly reduce embryogenesis, but higher levels of the inhibitor reduced (1.0 mM) and blocked (5.0 mM) embryo induction (Fig. 2). Additions of putrescine, the

enzymatic product of ADC, restored embryogenesis to control levels in the 1.0 mM treatments, whereas putrescine did not restore embryo development when 5.0 mM DFMA was present. This high concentration of DFMA may have had secondary toxic effects because embryogenesis was not restored in these cultures even when higher levels of putrescine were added (> 0.1 mM, data not shown). DFMO was used to determine the role of ODC in wild carrot embryogenesis, and it did not significantly inhibit development except at higher concentrations (5.0 mM) where, as in the case of DFMA, it was judged to be toxic (Fig. 2). These data suggest that ODC does not have a major function in wild carrot embryogenesis.

DFMA had a dramatic effect on the free polyamine levels in the cultured cells, decreasing the concentration of putrescine below limits of detection in our analysis system, and causing over a ten-fold reduction in spermidine (Table I). The spermine titer rose significantly in the treated cultures, which has been noted by investigators working with DFMO. The addition of putrescine to the DFMA treated cultures, at levels which restored embryogenesis, increased putrescine and raised the spermidine level to that of untreated, control cells.

TABLE I

EFFECT OF DFMA ON WILD CARROT POLYAMINE LEVELS

Treatment	Putrescine	Spermidine	Spermine
		(nMoles/g fresh wt.)	
Control	890 ± 121	506 ± 46	66 ± 25
DFMA (1.0 mM)	N.D.[a]	39 ± 16	260 ± 38
DFMA (1.0 mM) + Putrescine (0.1 mM)	185 ± 18	463 ± 143	115 ± 18
DFMO (1.0 mM)	645 ± 69	415 ± 17	66 ± 10

[a]None detected.

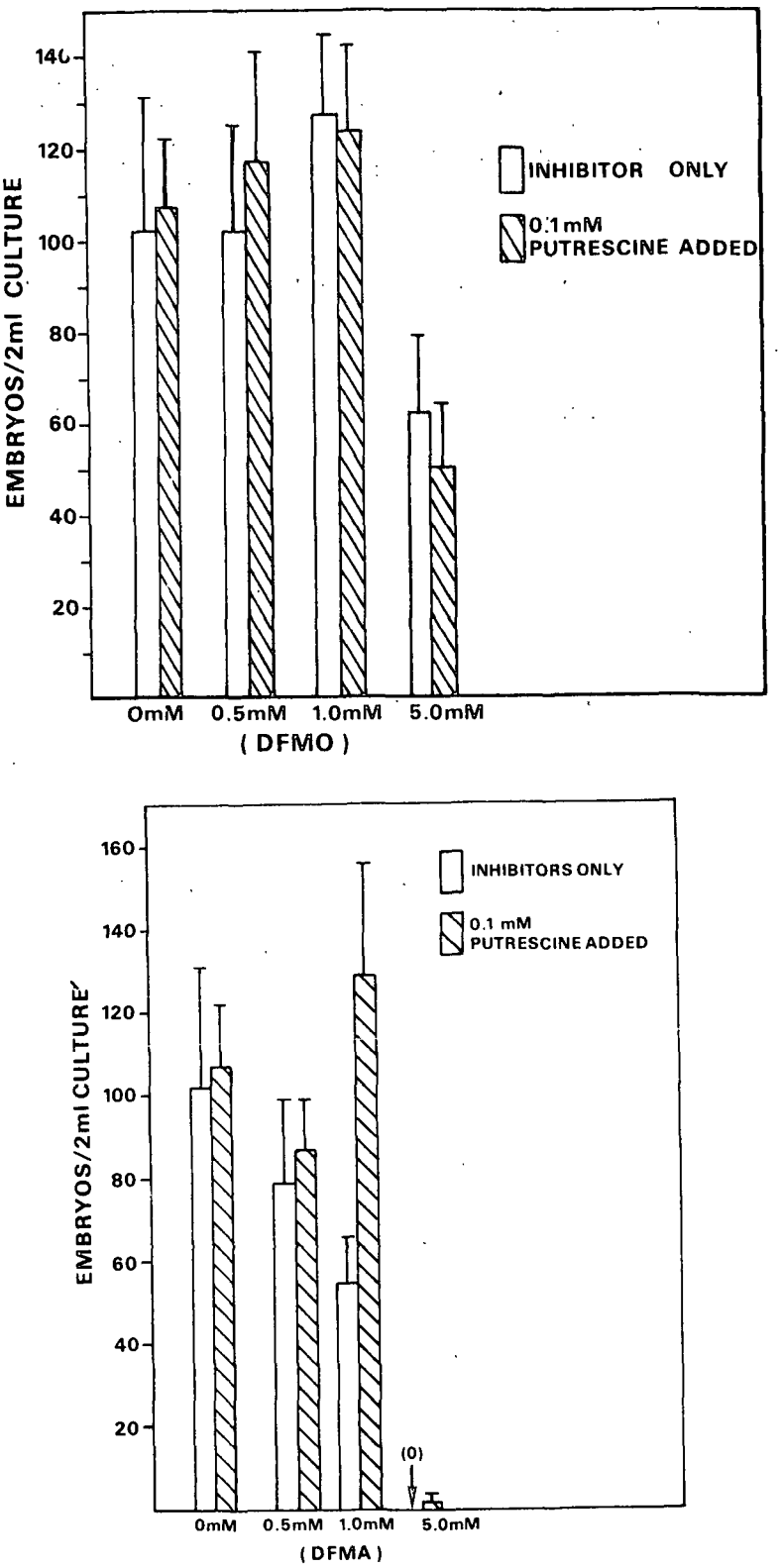


Figure 2. Effect of DFMA, DFMO, and putrescine on wild carrot embryogenesis.

In summary, we found that cultures treated with 1 mM DFMA exhibited nearly a 50% reduction in embryo formation as compared with controls, and additions of putrescine to the culture medium restored embryogenesis in the DFMA treated cultures. Putrescine and spermidine levels in the treated cells were dramatically reduced. Embryogenesis was not significantly affected by DFMO. These data suggest that polyamines have a major function in plant embryo development and that wild carrot synthesizes polyamines via the alternate biosynthetic pathway involving ADC rather than ODC.

Conclusions

The results of these experiments show that DFMA can effectively inhibit embryogenesis in suspension cultures of wild carrot. This inhibition of development by DFMA, which acts by decreasing ADC activity and lowering putrescine and spermidine levels in the cells, demonstrates that putrescine or spermidine are among the factors essential for embryogenesis. The importance of polyamines in development was also recently suggested in tobacco and tomato (4,5), as well as in animals where DFMO-induced polyamine depletion blocked embryo development in rats and rabbits (6).

Our studies also provide direct evidence that, of the two alternate pathways leading to polyamine biosynthesis in plants, the route involving ADC rather than ODC is involved in wild carrot embryo development. ODC has been shown to be associated with proliferative growth in plants, but preliminary data from several trials in our lab suggest that inhibition of ADC with DFMA affected only embryo differentiation and not culture growth as measured by fresh weight (data not shown).

ROLE OF SPERMIDINE IN WILD CARROT EMBRYOGENESIS

Introduction

Experiments on the effects of inhibitors of polyamine biosynthesis showed that DFMA lowered putrescine and spermidine levels in wild carrot cells. The resulting reduction of embryogenesis was reversed by additions of putrescine to the cells, suggesting that putrescine is required for embryo induction. Since DFMA reduced spermidine as well as putrescine levels in the cells and since putrescine is normally converted to spermidine, it is possible that spermidine, rather than putrescine, is required for embryo formation. In this case putrescine could restore embryogenesis in DFMA treated cultures because it was converted to spermidine. This series of experiments was carried out to determine if spermidine or spermine, as well as putrescine, could restore embryogenesis to DFMA treated cultures. Two inhibitors of spermidine synthesis were also tested to determine their effects on wild carrot embryogenesis.

Methods

The effects of the inhibitors on embryogenesis were tested as described in the preceding study. The inhibitors and polyamines were added to the cultures at day 0 of culture growth. MGBG (methylgloxal-bis guanylhydrazone) and DCHA (dicyclohexylammonium sulfate) are thought to block spermidine synthesis by inhibiting spermidine synthase and SAM decarboxylase, respectively, (2). DFMA was used only at 1 mM, a concentration which consistently causes about a 50% reduction of embryogenesis in our cultures (see previous experiments). The polyamines were all added at 0.1 mM.

Results

As expected, DFMA (0.1 mM) alone caused a reduction in embryogenesis. The data in Fig. 3 illustrate that additions of putrescine, spermidine, or spermine were equally effective in restoring embryogenesis in the treated cultures. When added to the cultures in the absence of DFMA, the polyamines had no significant effect on embryo formation.

Both MGBG and DCHA reduced embryogenesis in preliminary experiments (Fig. 4 and 5). These inhibitors, however, had similar effects on growth as measured by fresh weight gain.

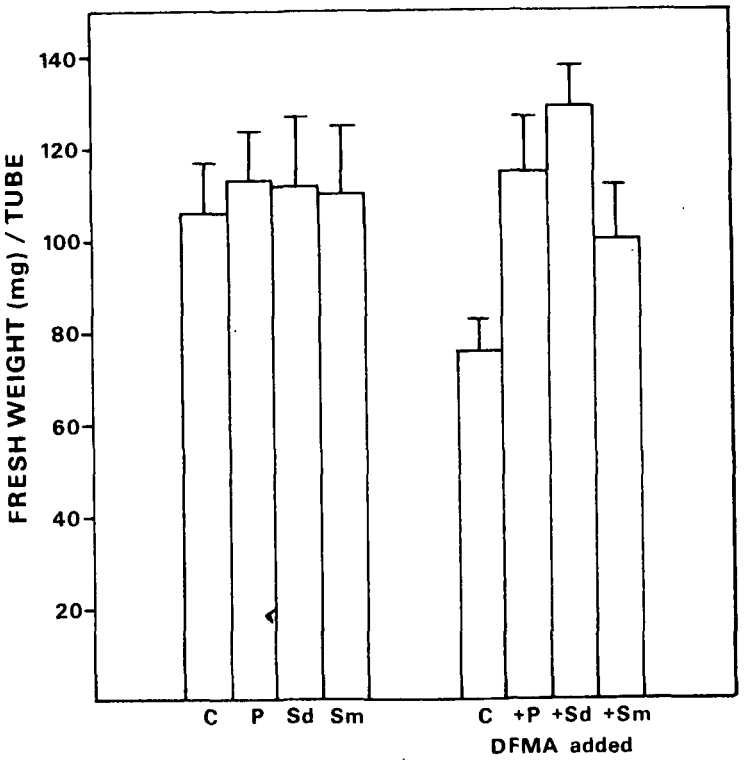
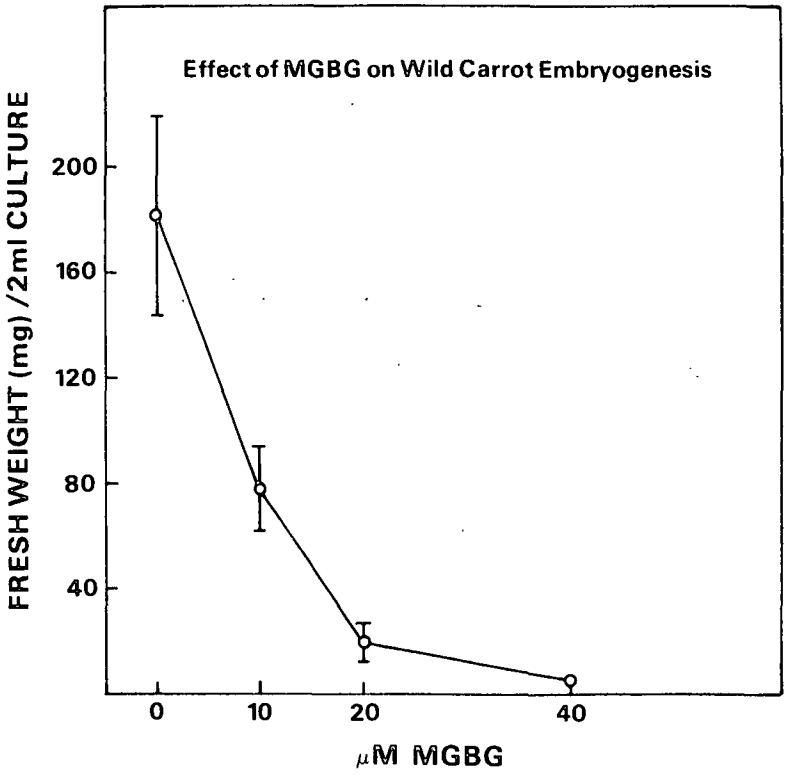


Figure 3. Effect of DFMA (0.1 mM) and polyamines on wild carrot embryogenesis.



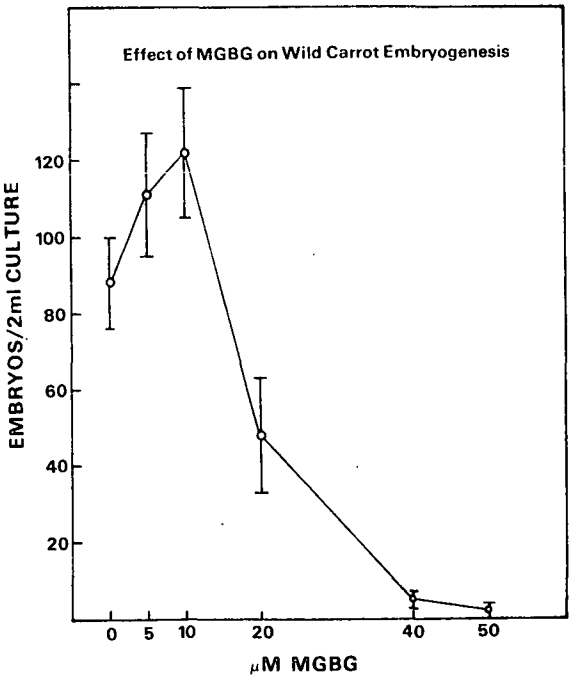
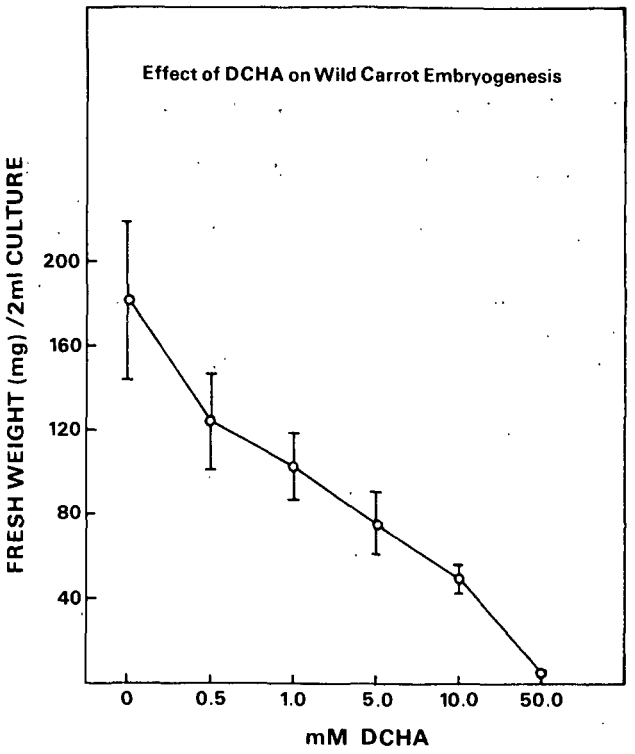


Figure 4. Effect of MGBG on wild carrot growth and embryogenesis.



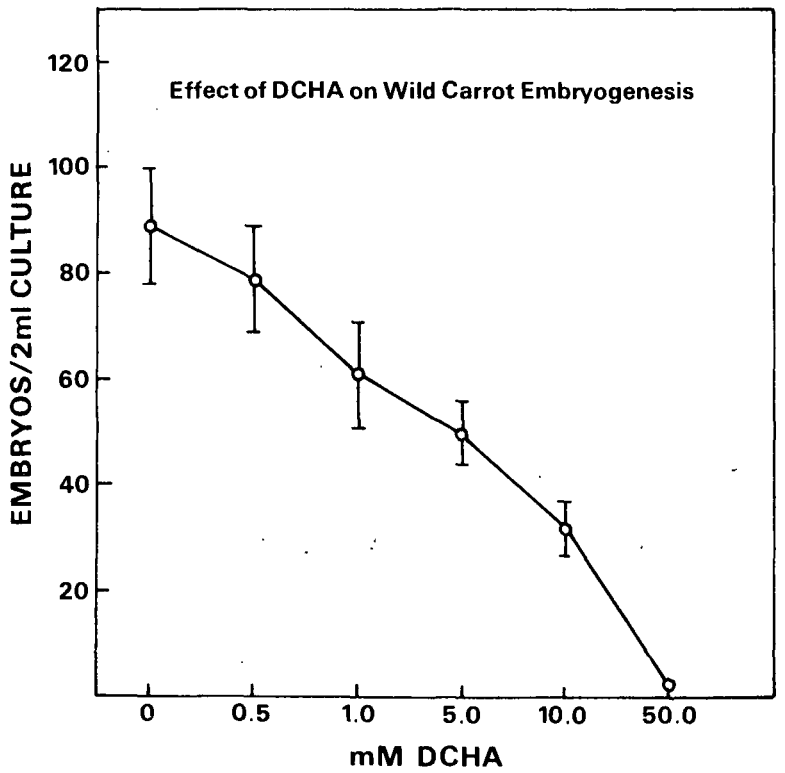


Figure 5. Effect of DCHA on wild carrot growth and embryogenesis.

Conclusions

Previous experiments have shown that DFMA lowers both putrescine and spermidine levels in the cells, without reducing spermine concentration. Those results suggest that spermine may not be important to embryogenesis. The present studies, in which spermidine alone restored embryogenesis to DFMA treated cultures and inhibition of spermidine biosynthesis with DCHA or MGBG blocked embryogenesis, suggest that spermidine is important in wild carrot development. Spermidine, rather than putrescine, may be the polyamine most required in wild carrot. Metabolic interconversions of the polyamines back to putrescine are possible and may account for these results; but other workers have shown that spermidine can serve as a polyamine source in ODC-deficient animal cells, and spermidine levels may be more closely correlated to growth in bacteria than is putrescine (7). Although not supported by a wealth of data, Kaur-Sawhney et al., have suggested that spermidine and its biosynthetic enzyme spermidine synthase may be physiologically important in a plant (oat) system (8). The importance of polyamines (spermidine) was also recently demonstrated in tobacco plants having altered polyamine metabolism (9). Mutant plants which exhibited altered morphologies, were regenerated from a cell line resistant to MGBG, the spermidine synthesis inhibitor.

This area of polyamine metabolism will be the subject of further studies in our lab. Obvious experiments include testing the ability of spermidine to restore embryogenesis in MGBG or DCHA treated cultures, determining the effects of these inhibitors on polyamine levels, and studying the enzymes involved in spermidine biosynthesis.

POLYAMINES DURING NATURAL PINE EMBRYO DEVELOPMENT

Introduction

Rises in polyamine levels and the associated biosynthetic enzymes have been associated with natural pine embryo development as well as wild carrot embryogenesis. Earlier progress reports have outlined free amino acid changes during ovule development of several pine species, in which relatively high levels of arginine were noted. Progress Report Ten (1983) contained data related to polyamine metabolism which showed that the levels of free polyamines rise at (or about) the time of ovule fertilization in white pine. Rises in levels of ODC and ADC, although not as dramatic as in wild carrot, were also noted.

Although polyamine metabolism cannot be studied during natural pine embryo development with the degree of sophistication possible with the model wild carrot system (i.e., it is not practical to use inhibitors), the 1982 data did suggest that polyamines are involved in pine embryogenesis. Cones were collected during the 1983 growing season in an attempt to reproduce the data previously collected and presented.

Methods

Developing pine cones were collected from red pine trees growing in a plantation located in an adjoining county. Collections were made once or twice a week, depending upon the suspected stage of cone development. Ovules excised from the developing cones were then collected for polyamine and enzyme analysis. Polyamine and enzyme assays were performed as described for wild carrot tissues.

Results

Changes in polyamine levels and ADC/ODC activity in developing white pine ovules were reported in Report Ten (1983). Polyamine data from red pine ovules collected during 1982 and 1983 appear in Fig. 6. The data represent the mean of three determinations. Although not shown, enzyme activity showed minor variations in levels over the time course of the study. The enzyme fluctuations were even less dramatic then those reported in Report Ten (1983) for white pine.

Conclusions

In general, data collected from red pine during the 1982 and 1983 growing seasons correlated well with the white pine. Polyamines rose at times that we suspected fertilization and embryo development to occur. Especially interesting are the data concerning spermidine. Spermidine rose more than either putrescine or spermine to become the dominant free polyamine present during embryo development. These data are of interest in light of our findings with wild carrot where spermidine was shown to be important to embryogenesis.

The free amino acid and polyamine data that have been collected thus far suggest that polyamines and their biosynthetic enzymes play a major, if not an essential role in the development of pine embryos in maturing cones. Although we cannot easily manipulate the natural, in vivo embryo development on the tree as we can with the wild carrot system, it may be possible to further study polyamine metabolism and other biochemical parameters during pine embryogenesis in the coming growing seasons.

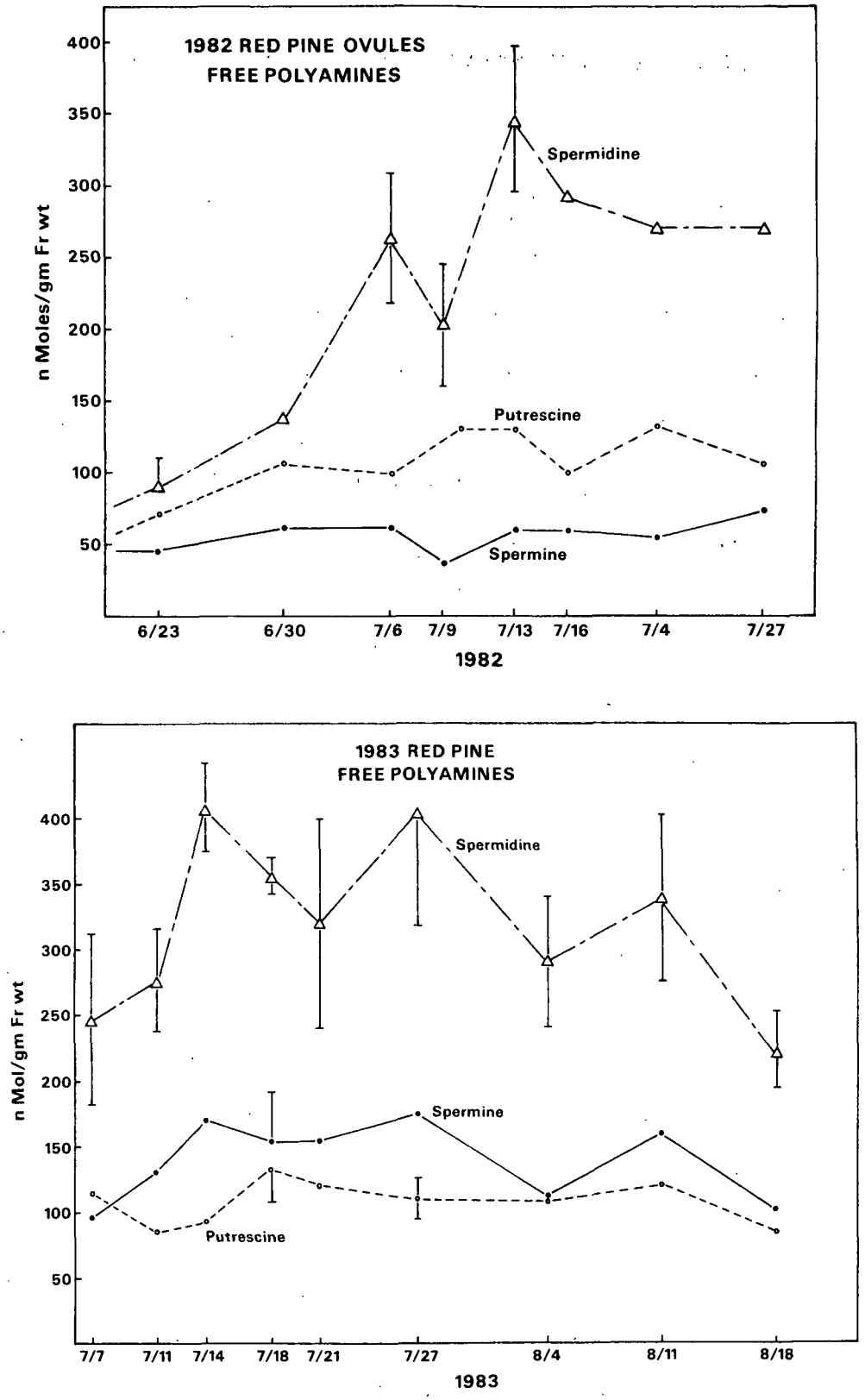


Figure 6. Free polyamine levels during embryo formation of red pine (1982 and 1983 growing seasons).

INFLUENCE OF NATURAL PINE CONE EXTRACTS ON WILD CARROT EMBRYOGENESIS

Introduction

As projected in the Plans section of last years' report, some extracts of developing pine seeds were made and tested as "undefined media." The main purpose of this avenue of research is to uncover, if they exist, any unrecognized growth and development factors used by the natural pine seed embryos during their development. Of course, such investigations may also turn up well-known factors, in which case the major effect might be to alter our priorities of planned growth regulator investigations. The approach taken was to test the effects of these extracts on wild carrot embryogenesis as an initial screen and then, providing no toxicity was observed, to use the extracts on the conifer cell suspensions. The latter aspect is not ready for inclusion in this report, but some actual stimulation of somatic embryogenesis in wild carrot was found as detailed below.

Materials and Methods

The extraction procedure consisted of preparing acetone powders of immature (just prior to or at fertilization) seeds dissected from loblolly pine cones. The resulting acetone phase was concentrated under nitrogen gas until a small amount of cellular water remained, which was then freeze-dried. After freeze-drying, weighed residue was dissolved in 0.05M potassium phosphate buffer (pH 6.8) to the extent possible and used as the low molecular weight addback fraction. Material water-insoluble at this stage has not been tested nor has any further fractionation of the water-soluble low molecular weight fraction been attempted. In addition to the foregoing fraction, weighed acetone powder was also extracted with 0.05M potassium phosphate buffer (pH 6.8) to yield the

high molecular weight addback fraction. Again, no further fractionation has been attempted.

The low molecular weight fraction in phosphate buffer was subjected to serial dilutions with phosphate buffer to obtain a series of extract concentrations. The usual test system consisted of wild carrot cells in either WCM or LM (see tabulated data) without 2,4-D (i.e., embryogenesis conditions). Tests were conducted in the dark in roller tubes with 2 mL of basal medium plus 0.1 mL of extract of various concentrations in phosphate buffer. Each treatment normally had five replicates. Controls without extract and with phosphate buffer replacing the extract were used. Evaluations consisting of visual inspection, fresh weights, and embryo counts were conducted at two or three weeks depending upon the growth rate of controls. Embryo counts by different observers resulted in the same conclusions concerning treatment effects.

Results

The effects of high and low molecular weight fractions of immature loblolly pine seed extracts upon wild carrot embryogenesis are shown in Tables II and III. An incompetent wild carrot cell line[1] was also tested in conjunction with the experiment that yielded the data of Table II; no embryos were generated in any controls or treated cells in that case. The data in Table II corroborate the findings of an earlier experiment (not presented).

Discussion and Conclusions

At this point there is little doubt that a presumably high molecular weight constituent(s) of immature loblolly pine seeds promotes wild carrot

[1]A cell line that grows well but does not produce embryos when 2,4-D is removed.

TABLE II

EFFECT OF HIGH MOLECULAR WEIGHT FRACTION OF LOBLOLLY PINE SEED EXTRACTS UPON WILD CARROT SOMATIC EMBRYOGENESIS

Test System	Response[a]	
	Embryo Count[b]	Fresh Weight, mg
Wild carrot in LM (day 14)		
Control	260 ± 28 (134 ± 12)	178 ± 20
Control + phosphate	126 ± 26 (22 ± 7)	19 ± 2
Control + extract in phosphate	338 ± 27 (200 ± 23)	200 ± 35
Wild carrot in WCM (day 21)		
Control	157 ± 17 (28 ± 7)	37 ± 6
Control + phosphate	339 ± 41 (154 ± 20)	261 ± 87
Control + extract in phosphate	487 ± 37 (215 ± 35)	306 ± 70

[a]Means ± SD of five replicates.
[b]Counts of second observer, who counted only structures $\geq$ 2 mm long, are in parentheses.

TABLE III

EFFECT OF LOW MOLECULAR WEIGHT FRACTION OF LOBLOLLY PINE SEED EXTRACTS UPON WILD CARROT SOMATIC EMBRYOGENESIS

Test System	Response[a]	
	Embryo Count[b]	Fresh Weight, mg
Wild carrot in LM (day 14)		
Control	16 ± 6 (11 ± 4)	21 ± 3
Control + phosphate	30 ± 6 (24 ± 4)	31 ± 6
Control + extract in phosphate		
full strength	0 ± 0 (0 ± 0)	0 ± 0
0.1 strength	43 ± 11 (30 ± 7)	12 ± 2 (n=3)
0.01 strength	45 ± 13 (32 ± 16)	26 ± 4
0.001 strength	42 ± 22 (33 ± 24)	28 ± 5 (n=4)

[a]Means ± SD of five replicates unless noted otherwise.
[b]Counts of second observer are in parentheses.

embryogenesis. This is apparent in the data of Table II and was also seen in the experiment mentioned above. The data are also of interest from two other points of view. First of all, the data demonstrate that different observers, even using different embryo-scoring criteria, came to essentially the same conclusions on embryo counts for treated versus control cells, i.e., although absolute values are quite different, the results would be similar if expressed as % of control. Another aspect that is evident in the data of Table II is the difference in the growth of wild carrot in LM versus WCM. Because WCM is actually phosphate limiting for wild carrot embryogenesis, a large increase in the number of embryos is seen due to phosphate addition with another 50% increase due to the high molecular weight extract fraction. The LM medium is not deficient in phosphate for wild carrot embryogenesis, and, therefore, the addition of phosphate to LM is inhibitory and causes a reduction in the number of embryos produced. For this reason, the positive effect of extract in LM is even greater, since the real control for the 338 value is 126, i.e., the extract overcame phosphate inhibition and had a further positive effect.

The case for the low molecular weight extract fraction is not clear. One reason for this is that the wild carrot cell line was losing competence at the time that the data of Table III were gathered. This can be appreciated by noting, relative to Table II, the low embryo counts in the controls and the absence of the negative phosphate effect in LM that was just described. Another factor to consider is that inhibitory substances are present in these extracts. This toxicity is easily seen in that no embryos formed when the extract was used "full strength," and, even at "0.1 strength" there is a negative effect on weight if not on embryo numbers. Thus, one has to go to "0.01 strength" to avoid toxicity, but at this dilution any positive factors are also quite dilute.

Nevertheless, given that the whole test system is questionable, the results are not as definitive as one would like. Rejuvenation of the wild carrot cell line is in progress and this aspect of the testing should present no serious problems in the future.

The toxicity of the low molecular weight fraction is also of concern. One approach might be to extract ovules only (as opposed to whole immature seeds), but, as noted in previous discussions, the dissection problems involved make this approach impractical. Another way to attack the problem would be to further fractionate the low molecular weight fraction, which we will probably attempt. The data suggest that the toxicity is not due to something as simple as phosphate.

The next logical step in testing the high molecular weight fraction will be to use it with conifer cells. Beyond that, both high and low molecular weight fractions do appear to require some further resolution before additional testing. For the low molecular weight fraction, this means minimally the removal of toxic components. To the extent that positive responses continue, it will be necessary to seek the identity of responsible component(s). There is also the question of whether to test the water-insoluble components.

GROWTH AND EMBRYOGENESIS OF WILD CARROT CELL SUSPENSIONS IN LM (Explant Source, Somatic Embryos)

Introduction

In last year's report we reported that a competent wild carrot cell suspension could be maintained in LM, and that embryogenesis could be induced with the same cells in LM. However, in any somatic embryogenesis tissue culture system there are three distinct phases: (1) initiation, (2) maintenance, and (3)

induction. Last year's work was only relevant in that it proved that LM could be used to maintain a competent cell suspension and induce embryogenesis (therefore only the last two phases of the tissue culture system were tested). Thus a question still remained as to the usefulness of LM in inititating competent cell lines. A study was undertaken to answer this question.

Methods

Wild carrot somatic embryos growing in wild carrot (WC) medium were used as the explant source. Ten somatic embryos were placed on each of the 13 different agar media treatments found in Table IV. All media treatments contained 0.5 ppm 2,4-D. Calli derived from the treatments were subcultured into liquid medium of the same composition in order to obtain a liquid cell suspension. For the control treatment (wild carrot medium), each callus derived from a somatic embryo was treated as a separate line. For all other treatments all the calli from that treatment were pooled together to produce a single cell suspension or a single line.

Once the cell suspensions were obtained the cells were launched (Embryogenesis Trial I) using standard protocol (0.5 µL screened, washed cells/mL; medium minus growth regulator, using five 2-mL volumes in roller drum tubes). When the roller drum tubes were harvested at 14 to 21 days, fresh weights and embryo counts were recorded for each tube. A second launch (Embryogenesis Trial II) was conducted using the same cells and experimental protocol but only after the cells had been in liquid culture for a prolonged time (6 months). This was done to test the ability of the medium to maintain a culture over a prolonged period of time.

TABLE IV

THE INFLUENCE OF LM-MEDIA COMPONENTS ON INITIATION, MAINTENANCE AND SOMATIC EMBRYOGENESIS OF WILD CARROT

In each medium, calli from 10 embryos were pooled and rendered into suspensions

	Embryogenesis I[a]			Embryogenesis II[a]		
		Embryo No.			Embryo No.	
Medium	Growth, mg D.wt. ± SD	Total, ± SD	Plantlets,[b] %	Growth, mg D.wt. ± SD	Total, ± SD	Plantlets,[b] %
1 (WC)	10.2 ± 1.6	94 ± 21	34	5.2 ± 0.2	15 ± 3	13
2 (Su↑)	7.1 ± 0.4	14 ± 4	24	10.5 ± 0.9	18 ± 7	24
3 (PO_4↑)	13.3 ± 0.6	22 ± 12	15	12.4 ± 0.8	146 ± 45	42
4 (NO_3↑)	9.6 ± 2.1	29 ± 24	9	8.1 ± 0.4	1 ± 1	0
5 (2,3,4)	14.0 ± 1.0	7 ± 6	0	16.1 ± 0.9	10 ± 4	10
6 (Mg↑)	5.8 ± 1.9	76 ± 9	6	3.6 ± 4.1	16 ± 13	44
7 (Ca↓)	Little growth	--	--	--	--	--
8 (6+7)	4.1 ± 0.9	3 ± 3	6	Little growth	--	--
9 (5+8)	15.8 ± 0.45	33 ± 8	31	21.7 ± 0.4	148 ± 10	70
10 (Micros↑)	15.1 ± 1.3	182 ± 11	62	13.8 ± 2.0	75 ± 4	55
11 (5+10)	12.1 ± 1.6	19 ± 21	12	22.1 ± 2.8	26 ± 17	23
12 (8+10)	Little growth	--	--	--	--	--
13 (LM)	4.3 ± 6.9	7 ± 8	51	16.1 ± 4.2	91 ± 5	49

[a] Embryogenesis I denotes embryo induction at 3 months involving 2 transfers of cultures on solid media and one in liquid media. Embryogenesis II denotes induction after 5 additional biweekly subcultures in liquid media.

[b] % Plantlets are the % embryos greater than 5 mm in length and exhibiting both root and Cotyledon development.

Results

Tables IV and V summarize the results of Embryogenesis Trial I and Embryogenesis Trial II.

Discussion

Somatic embryos were used as an explant source because they should represent a homogeneous starting material. Table V shows the amount of variability that was exhibited by the control treatment (WC medium) using somatic embryos as the explant source. The most important points to be obtained from

Table V are (1) there is quite a bit of variability in the controls and (2) competency seems to decrease with time with this treatment.

TABLE V

RESPONSE OF CULTURES ORIGINATING FROM SINGLE SOMATIC EMBRYOS OF WILD CARROT

	Embryogenesis I			Embryogenesis II		
		Embryo No.			Embryo No.	
Culture (from single embryo)	Growth, mg D.wt. ± SD	Total, ± SD	Plantlets,[a] %	Growth, mg D.wt. ± SD	Total, ± SD	Plantlets,[a] %
1	9.0 ± 0.9	69 ± 14	37	10.0 ± 2.0	41 ± 18	29
2	7.2 ± 0.3	38 ± 7	16	8.0 ± 0.7	43 ± 10	23
3	4.9 ± 0.7	25 ± 16	17	15.6 ± 1.2	42 ± 4	50
4	11.8 ± 1.4	170 ± 24	23	15.2 ± 5.2	101 ± 14	42
5	11.5 ± 1.1	145 ± 16	14	17.4 ± 3.3	87 ± 3	49

[a]% Plantlets are the % embryos greater than 5 mm in length and exhibiting both root and Cotyledon development.

In Table IV the calli from the 10 somatic embryos of each treatment were pooled to produce one liquid suspension; thus the standard deviations represent the error in putting out the 5 replications of the experiment. Because of the high variability encountered in the control experiment (Table V) there are no statistically significant differences among the thirteen different media. However, there are some important conclusions that can be obtained from this experiment:

(1) LM was used to initiate a competent cell line from somatic wild carrot embryos.

(2) LM was used to maintain competent wild carrot cells with results that were better than for the wild carrot medium (Launch I results compared to Launch II results).

(3) Certain media changes (7,8,12) are catastrophic.

(4) Certain media changes (3,9,10,13) appear to be beneficial at either the initiation (agar) or maintenance (liquid) level.

(5) Some medium changes (2,5,11) seem to preferentially increase growth over embryogenesis.

GROWTH AND EMBRYOGENESIS OF WILD CARROT CELL SUSPENSIONS IN LM (Explant Source, Wild Seed)

Introduction

It was previously shown that it was possible to initiate, maintain and induce embryogenesis in wild carrot if somatic embryos were used as the explant source. In our own trials with gymnosperms, excised mature embryos from seeds were used as the explant source. Thus a question was raised as to the ability of LM to initiate competent cell lines using mature embryos from wild carrot seed as the explant source. An experiment was run to answer this question.

Methods

Wild seed from some wild carrot plants growing in our area were harvested. Ten seedlings produced from wild seed were placed on each of two media treatments (WC and LM), containing 0.5 ppm 2,4-D. Calli from each seedling were obtained and placed in liquid culture. From the resulting liquid suspensions, launch or embryogenesis trials were conducted using the standard protocol (0.5 µL screened, washed cells/mL; medium minus growth regulator, using five 2 mL volumes in roller drum tubes). After 14-21 days the tubes were harvested, and fresh weight and embryo numbers were determined.

Results

The results from this experiment are summarized in Table VI.

TABLE VI

EMBRYOGENESIS TRIALS WHEN SEEDLINGS FROM WILD SEED WERE USED AS THE EXPLANT SOURCE

	Medium	
	WC	LM
Means[a]	48	34
Std. dev. ±	36	43

[a]Embryo numbers per 2 mL, based upon 5 replications.

Discussion and Conclusions

LM can be used to initiate, maintain, and induce competent wild carrot lines when seedlings from wild seed are used as the explant source. There appear to be no significant differences between wild carrot medium and LM in this experiment[1].

Of the four major variables that we encounter in tissue culture, (1) explant source, (2) medium, (3) growth regulator, and (4) light, medium does not appear to be the limiting variable in our obtaining competent cell lines.

EFFECT OF CONE STORAGE ON FREE AMINO ACIDS, POLYAMINES, AND ENZYME ACTIVITY

Introduction

A major reason that polyamines, amino acids, etc., were studied in red/white pine rather than in developing loblolly pine cones involves the geographic location of the trees. Loblolly pine samples must be shipped to Appleton and, even under the best of circumstances, may spend a day or two in transit. Tissues (cones) collected from red or white pine, however, are

[1]The high variability in this experiment was expected and can be explained by the fact that wild seed should be more variable than somatic embryos.

available locally and can be transported to the lab in less than an hour. Since we could only speculate on the effects of shipping on cones sent from a distant source, this study was carried out to experimentally determine the effects of this handling.

Methods

Red pine cones collected and handled as described in a previous experiment were considered "fresh" samples. A number of cones were placed into plastic bags and stored in a refrigerator for three days to simulate transit conditions. Free amino acid, polyamine, and enzyme analyses were performed on these and the fresh cones.

Results

The effects of storage on the free amino acid pools is illustrated in Table VII. Although most of the changes in levels may not be statistically significant, levels of many amino acids tend to increase during storage.

TABLE VII

EFFECT OF STORAGE ON FREE AMINO ACIDS
µmoles/g fresh weight

	Fresh	Stored		Fresh	Stored
asp	0.49 ± 14	0.86 ± 0.32	cys	t	t
hyp	N.D.	N.D.	met	0.33 ± 0.13	0.15 ± 0.03
thr	0.34 ± 0.14	0.23 ± 0.07	ile	0.19 ± 0.02	0.14 ± 0.10
ser	1.17 ± 0.31	0.75 ± 0.09	leu	0.22 ± 0.04	0.14 ± 0.10
asn	N.D.	N.D.	tyr	0.21 ± 0.12	0.07 ± 0.07
gln	15.70 ± 4.33	27.5 ± 10.89	phe	0.14 ± 0.07	0.06 ± 0.4
glu	6.88 ± 0.81	7.43 ± 1.57			
pro	0.77 ± 0.03	1.85 ± 0.31	γ-aba	0.33 ± 0.31	0.14 ± 0.24
gly	0.29 ± 0.02	0.28 ± 0.12	trp	0.35 ± 0.33	0.73 ± 0.79
ala	3.58 ± 0.87	2.47 ± 0.20	orn	0.33 ± 0.10	0.20 ± 0.18
val	0.29 ± 0.17	0.23 ± 0.07	lys	0.69 ± 0.26	0.77 ± 0.53
			his	0.90 ± 0.41	1.34 ± 1.11
arg	6.26 ± 1.65	9.33 ± 6.29			
	Total	39.51		54.70	

With respect to polyamine metabolism, it appears that only putrescine changed significantly during storage (Table VIII). Putrescine levels nearly doubled in the tissues, while spermidine, spermine, and the enzymes ADC and ODC did not significantly rise or fall.

Conclusions

It was felt that our simulated storage protocol was a valid approximation of shipping conditions. Samples received in our lab have often been in transit for 2-3 days, and only rarely have samples been received in a cold (<5°C) state. Although changes in the biochemical parameters that were measured were not as dramatic as might be predicted, it is clear that shipping of cones (or any samples) for later biochemical analysis should be done with caution. Free amino acids generally rose in the stored samples, and this might be the expected result of protein breakdown/turnover. Putrescine levels in the samples were affected the most of any parameter measured, and this also was not unexpected. Putrescine elevations are considered to be valid, sensitive indicators of pH and osmotic stress in plant tissues (10).

TABLE VIII

EFFECT OF STORAGE ON POLYAMINES AND SELECTED ENZYMES

Polyamine	Fresh (n mole/g fr. wt.)	Stored (n mole/g fr. wt.)
Putrescine	132 ± 24	258 ± 30
Spermidine	356 ± 13	340 ± 30
Spermine	153 ± 30	152 ± 18
Enzyme Activity	**(n mole CO_2/g fr. wt. · hr)**	
ADC	0.6 ± 0.1	0.7 ± 0.2
ODC	0.9 ± 0.3	0.8 ± 0.2

It appears that cone storage and probably shipping do affect the biochemical state of pine tissues. This reinforces our feeling that biochemical monitoring of natural pine embryogenesis should be carried out on locally available trees.

EFFECT OF STORAGE OF EXPLANT SOURCE ON ORGANOGENESIS

Introduction

Because many of the gymnosperm species we are interested in are not indigenous to our region, we wondered about the effects of shipping on the cones sent to us, especially with regard to biochemical monitoring and the quality of the immature embryos as an explant source. Thus we attempted to answer the second of these questions with the following experiment.

Method

White pine and red pine cones were collected near Appleton. Immature embryos were dissected from these cones and 20 placed on each of 6 agar treatments (2 media x 3 growth regulators) for organogenesis trials. Some of the same cones were placed in a refrigerator for 3 days to simulate shipping. After 3 days, immature embryos were dissected from the stored cones and 20 placed on each of the same 6 treatments. Evaluation of the immature embryos took place at 1 and 3 months after they were initially cultured. The number of embryos transferred and the number of embryos successfully yielding organogenetically derived shoots were recorded.

Results

Table IX lists the 6 treatments employed and the number of explants transferred for each species and treatment. Embryos yielding organogenetically

derived shoots (data not given) were approximately equal to number of explants transferred.

TABLE IX

NUMBER OF EXPLANTS (OUT OF 20) TRANSFERRED IN TRIALS TO DETERMINE THE EFFECT OF STORAGE OR SHIPPING ON EXPLANT SOURCE

Species	MS 0.01 NAA/ 1BAP	LM 0.01 NAA/ 1BAP	LM 0.01 NAA/ 1BAP + PA[a]	MS 0.01 NAA/ 0.1BAP	LM 0.01 NAA/ 0.1BAP	LM 0.01 NAA/ 0.1BAP + PA[a]
Red Pine						
Fresh	3[b]	6	3	3	3	5
Stored[c]	2	4	2	5	3	4
White Pine						
Fresh	8	8	8	10	7	11
Stored	11	7	13	12	7	9

[a]Polyamines - 0.1 mM putrescine and 0.1 mM spermidine added.
[b]20 Reps or embryos cultured per treatment and species addition.
[c]Cones were stored for 3 days at refrigerator temperature (± 40°F).

Discussion and Conclusions

Storage (3 days refrigeration) had no significant effect on immature embryo explants as a source of tissue for organogenetic trials. Also, the polyamine additions and media investigated had no significant influence on obtaining organogenetically competent material.

We feel, however, that this experiment should be repeated with larger numbers of embryos. Also, we should look at the influence of long term storage on organogenesis.

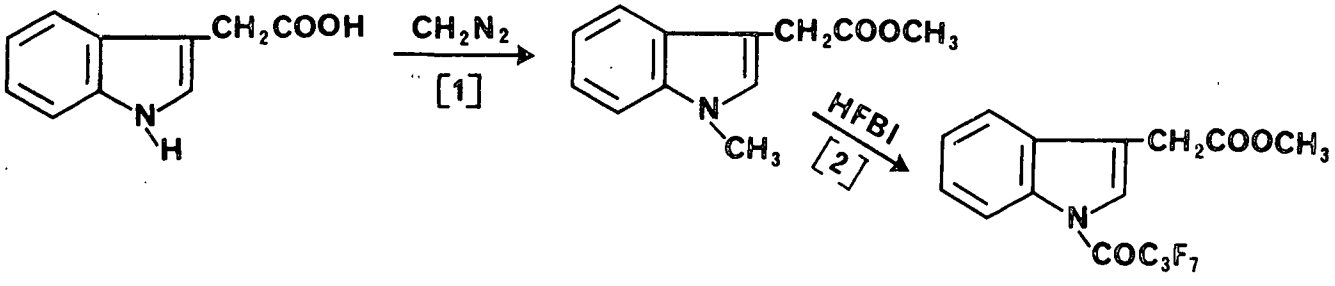
IAA LEVELS IN WILD CARROT

Introduction

It has long been recognized that it would be of great value to this project to acquire some quantitative data on levels of endogenous phytohormones in the cells of our model systems versus cultured conifer cells. Due principally to the minute quantities involved, research in this area can be very demanding. In Progress Report Six (p. 36) appears a small amount of data of this nature on IAA concentrations in Douglas-fir seedling needles and callus; these hard-won numbers were obtained by a tedious isotope derivative procedure. Recently, a modified version of that procedure which employs mass spectrometry as a final step was developed by Ike Andrews in his Ph.D. thesis research at IPC and applied by the research team to the analysis of IAA in wild carrot cell suspensions.

Materials and Methods

The selective ion monitoring (SIM) mode for quantitative analysis of IAA by gas chromatography/mass spectrometry (GC/MS) was adapted from the method of Rivier and Pilet (11). This analysis requires two steps in which derivatives of IAA are made as follows:



The product after reaction with diazomethane and then heptafluorobutyrylimidazole (HFBI) is large enough so that, in theory at least, it becomes easier to resolve from other interfering substances in the GC/MS technique. Once this derivative was made, the real time-consuming operations were over, and the SIM mode could be used to monitor ion fragments of 385 (molecular ion of the derivative) and 326 (base peak). In addition, C-14 labeled IAA was used as an internal standard for the extraction and workup phases; the recovery of the C-14 IAA can be monitored as a molecular ion of 387 or by scintillation counting. A flow diagram typical of the extraction, pre-purification, and derivatization procedures is given in the Appendix. The TLC step was omitted in obtaining the wild carrot data presented below.

Results

The IAA content of proliferative wild carrot cells growing in the presence of 2,4-D is presented in Fig. 7. The IAA levels for wild carrot cells forming embryos in medium lacking 2,4-D is shown in Fig. 8. Corresponding growth data appear in Fig. 9. The wild carrot cell line here is competent clone WC8-3 growing in wild carrot medium (WCM).

Discussion and Conclusions

The data for the wild carrot model presented in Fig. 7 and 8 is probably the best we have generated using this technique of IAA analysis. Since 500 mg samples are required, there is no replication in the data reported here except that the zero time point in the + 2,4-D data is a duplicate mean (23.5 ng/g fr. wt.) that in itself was not very reassuring. The 23.5 was an average of 17.1 and 30.0. Inspection of the raw data would show that the recovery of the internal standard was the ruling factor in the difference, being 56.3% for

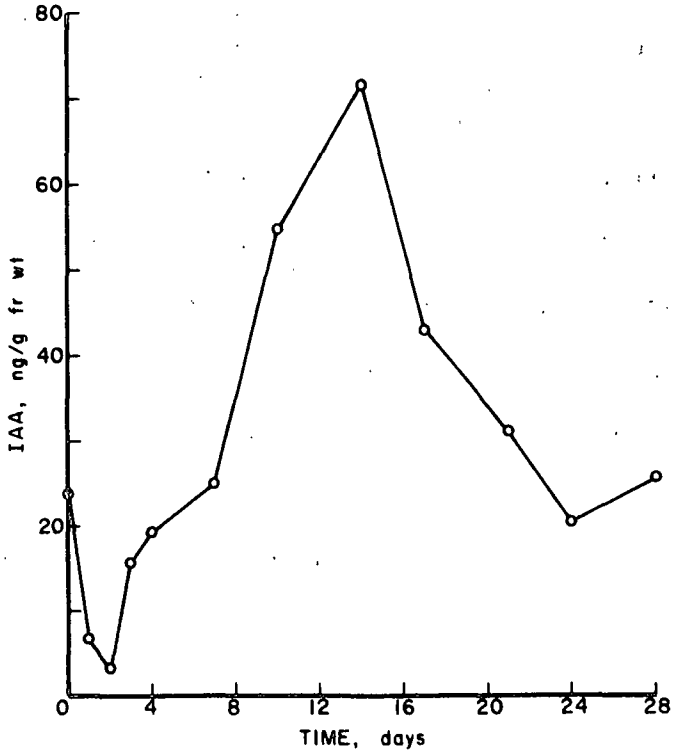


Figure 7. The IAA content of wild carrot suspensions cells as a function of time under proliferative (+ 2,4-D) conditions.

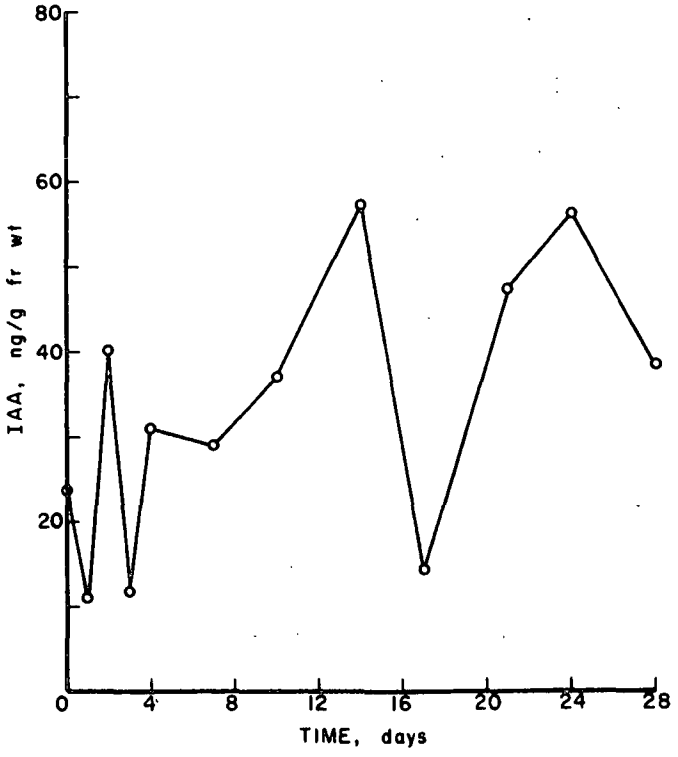


Figure 8. The IAA content of wild carrot suspension cells as a function of time under somatic embryogenesis (- 2,4-D) conditions.

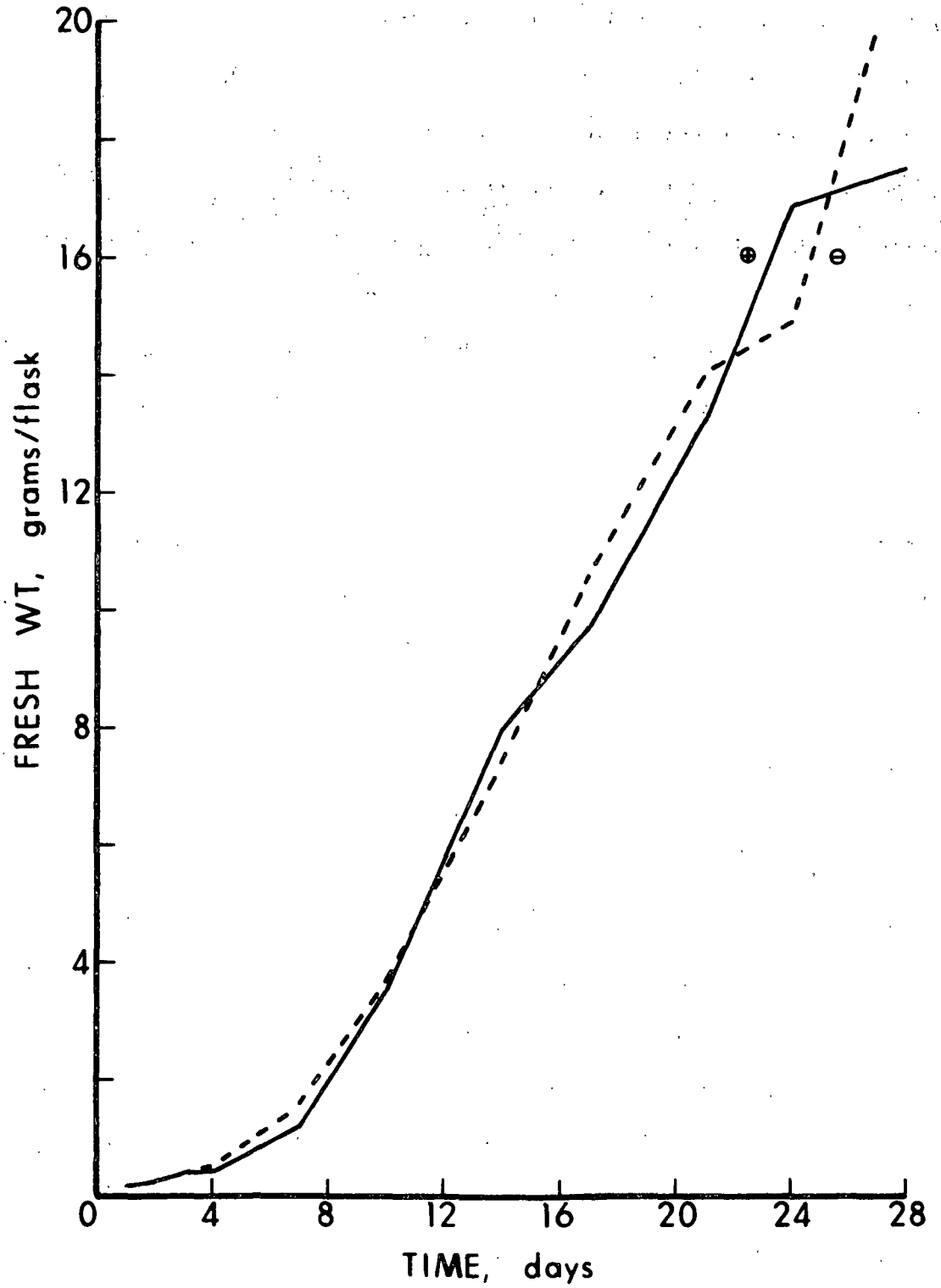


Figure 9. The growth curves for wild carrot suspension cells under + and - 2,4-D conditions, for which the IAA content was determined in Fig. 7 and 8.

the 17.1 value and 33.1% for the 30.0 figure. Given that recoveries for all other data points on the + 2,4-D graph ranged from 45.7% to 70.8%, the 30.0 value may represent an overcorrection for low recovery. On the basis of this reasoning, the samples at days 2, 21 and 28 were characterized by low recoveries in the - 2,4-D set and these three time points might be most suspect. Indeed, the point at day 2 on the - 2,4-D graph does seem to be out of line. The recoveries used for these graphs were determined by the scintillation counter method; the corresponding calculations based on the 387 molecular ion of C-14 IAA show similar trends.

It can be appreciated from the foregoing discussion that these data are not completely satisfying. Nevertheless, given these nagging shortcomings, it still appears reasonably certain that the cells of both the + and - 2,4-D sets begin with an initial drop in IAA for the first couple of days after transfer to fresh medium. This drop is then followed by a steady rise to a peak level of 60 to 70 ng/g fr.wt. at day 14 in both cases. The difference then emerges in that the - 2,4-D culture forming embryos shows a second rise in IAA level which is not seen in the + 2,4-D proliferative case. Inspection of the + and - 2,4-D growth curves in Fig. 9 shows that, at the time of the second IAA peak, the - 2,4-D embryo growth is continuing at a rapid pace after what may have been a brief hesitation. Meanwhile, the + 2,4-D cells, which exhibit no second IAA spike, are slowing down and entering stationary phase at this time. The reality of two IAA peaks is rendered somewhat more believable by similar trends for IAA in another set of data (not shown) obtained with another wild carrot cell line somewhat earlier. Nevertheless, a further confirmation of the second rise in IAA by line WC8-3 undergoing embryogenesis would be needed to be

reasonably confident of this interpretation. Unfortunately, obtaining these data strains our limited resources as already noted.

It is important to note that the IAA levels that we are reporting here would seem to be in line with what other investigators are finding. Perhaps the most relevant data is that of M.C. Elliott's group working with Acer pseudoplatanus cell suspensions. This system is more like our conifers than wild carrot in that it grows well in the presence of 2,4-D but goes nowhere when transferred to 2,4-D-free medium. Elliott (12) has published data for the + 2,4-D Acer suspensions which show a large spike of IAA at day 10; considering species and other operational differences, this is remarkably close to the day 14 initial spike seen in both + and - 2,4-D wild carrot suspensions. Unfortunately, Acer doesn't undergo somatic embryogenesis, so there appears to be no data on - 2,4-D systems. It should also be mentioned here that there is some data in the literature on the IAA content of Douglas-fir (13) and Pinus sylvestris (14) seedlings. Caruso (13) has recently revised IAA estimates for Douglas-fir downward by an order of magnitude. Some appreciation for the range of IAA content reported for various species, etc., can be obtained by examining Tables I and II in the publication of Sweetser and Swartzfager (15).

The loblolly pine suspension cells can also be analyzed for IAA by the GC/MS method, but we have found that the TLC step of the flow diagram can't be omitted in this case. The only thing that can be stated about loblolly pine cells (10-D Cot) as of this writing is that at "zero time" they contain nearly the same level of IAA as found for "zero time" wild carrot cells. This value in hand for 10-D Cot is 21 ng/g fr.wt. IAA analysis was also run in triplicate on the natural pine embryo model this past summer; however, even with the TLC step

included, many of these samples were too "dirty" at the final step to yield reliable data. At the present time it appears fruitless to attempt this analysis on natural pine model samples without further improvements in the technique.

It is tentatively concluded that embryogenesis in the wild carrot model is accompanied by a second increase in IAA content during more advanced stages of embryo development (e.g., 3 weeks). If this can be confirmed, it would be a useful though somewhat late and cumbersome biochemical marker that could also be expected to occur in conifer somatic embryogenesis. Perhaps just as importantly, it may help us to tie together interpretation of other aspects of embryogenesis known to be linked to endogenous auxin gradients or concentrations. A second burst of IAA might correspond with additional cell division in meristemoidal regions of the embryo which become activated in later stages of development.

OBJECTIVE I RESEARCH

PHOSPHATE UPTAKE STUDIES

Introduction

Previous media studies have shown that phosphate is important in cell growth and that the levels of phosphate in the medium can alter synthesis or breakdown of the free amino acid pool. The reason phosphate is so important is probably due to its central role in energy production of the cell.

It has been shown that embryogenesis and organogenesis are conditions requiring high energy and that most metabolism involves primary rather than secondary products. However, when we try to induce embryogenesis in our cultures, the cells produce large amounts of secondary metabolism products (tannins or phenolics). Thus we are concerned with the energy related metabolism of our systems. As a first approximation we have monitored the phosphate uptake of the wild carrot system under "plus" growth regulator (proliferative) and "minus" growth regulator (embryogenesis) growth conditions, and of our own 10-D Cot line.

Methods

Wild carrot cells were placed into 10 mL roller drum tubes with the following conditions:

(1) "plus" growth regulator, 0.5 µL/mL I.D., wild carrot medium[1]

(2) "minus" growth regulator, 0.5 mL/mL I.D., wild carrot medium

(3) "minus" growth regulator, 0.5 µL/mL I.D., LM medium.

The 10-D Cot line was placed into 10 mL roller drum tubes with the following treatments:

[1]The growth regulator was 2,4-D and I.D. refers to the inoculation density.

(4) "plus" growth regulator, 10 µL/mL I.D., LM medium

(5) "plus" growth regulator, parental I.D., LM medium

(6) "minus" growth regulator, 10 µL/mL I.D., LM medium

The procedure used was to measure growth in the roller drum tubes at days 1, 2, 3, 6...etc. At the times the tubes were harvested, dry weights and the phosphate concentration left in the medium were recorded. The method used for phosphate analysis can be found in the Appendix (Table XXIV).

Results

Figures 10-15 show the phosphate removed (as a percent of total phosphate in the medium) vs. time for the six different culture conditions. Figures 10-15 also provide the growth curves for these cultures.

Discussion and Conclusions

Figures 10-12 for the wild carrot cultures show phosphate removal from the media to (1) precede growth or (2) to occur more rapidly than growth. Also the phosphate is depleted from the wild carrot medium by day 9. In the LM medium, growth is more nearly exponential and phosphate depletion does not occur until day 14. From days 14-17 phosphate concentration in the medium increases. This may be due to lysing of cells, (or simply the release of phosphorus by the cells), however, total mass (growth) is still increasing at this time.

With respect to the gymnosperm cell cultures, the results are as follows. For Fig. 13 and 14, phosphate uptake precedes growth. The parental inoculum depletes the phosphate around day 9, while an inoculum of 10 µL/mL does not deplete the phosphate until day 14. In both cases a reduction in mass results in an increase of phosphate in the medium. Thus the 10-D Cot

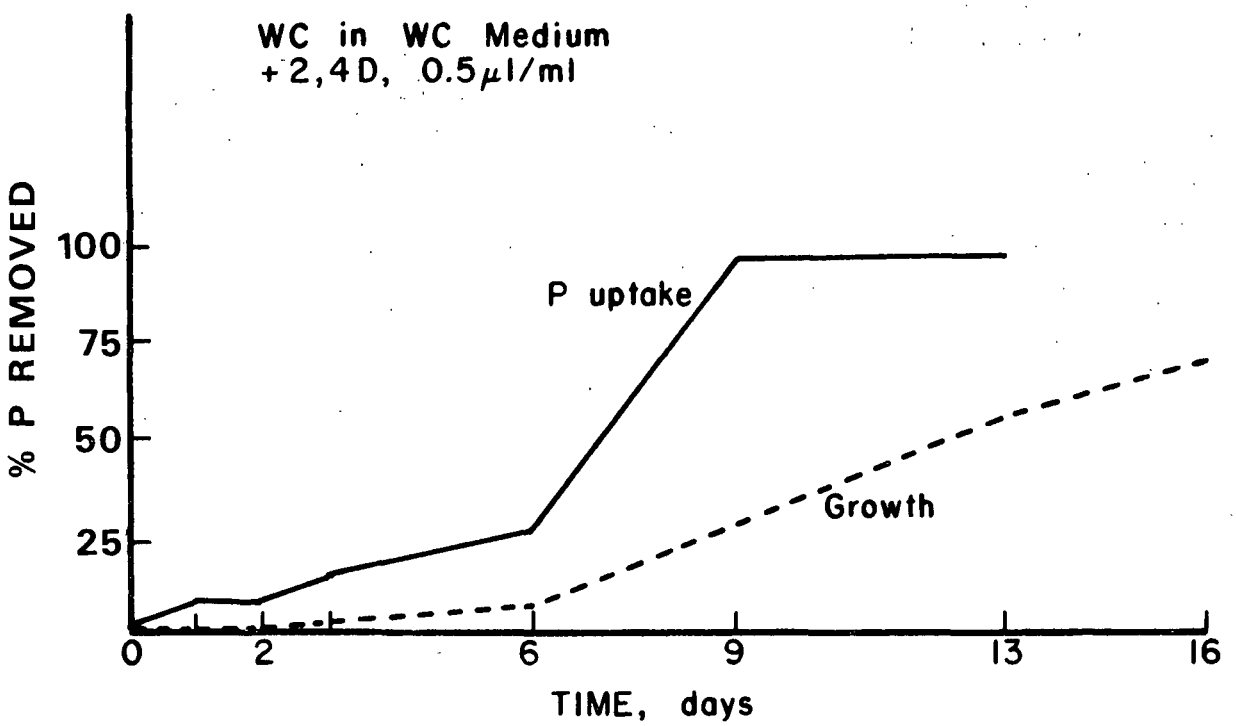


Figure 10. Phosphate uptake for wild carrot cells in wild carrot medium with 2,4-D.

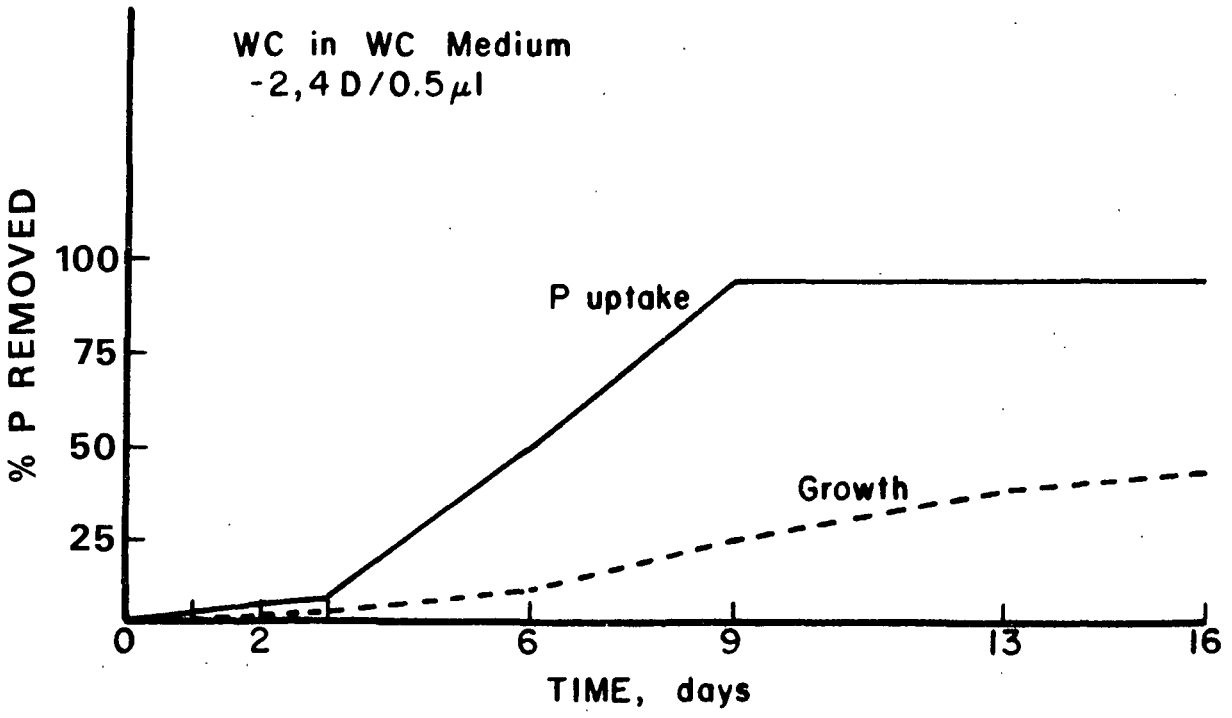


Figure 11. Phosphate uptake for wild carrot cells in wild carrot medium without 2,4-D.

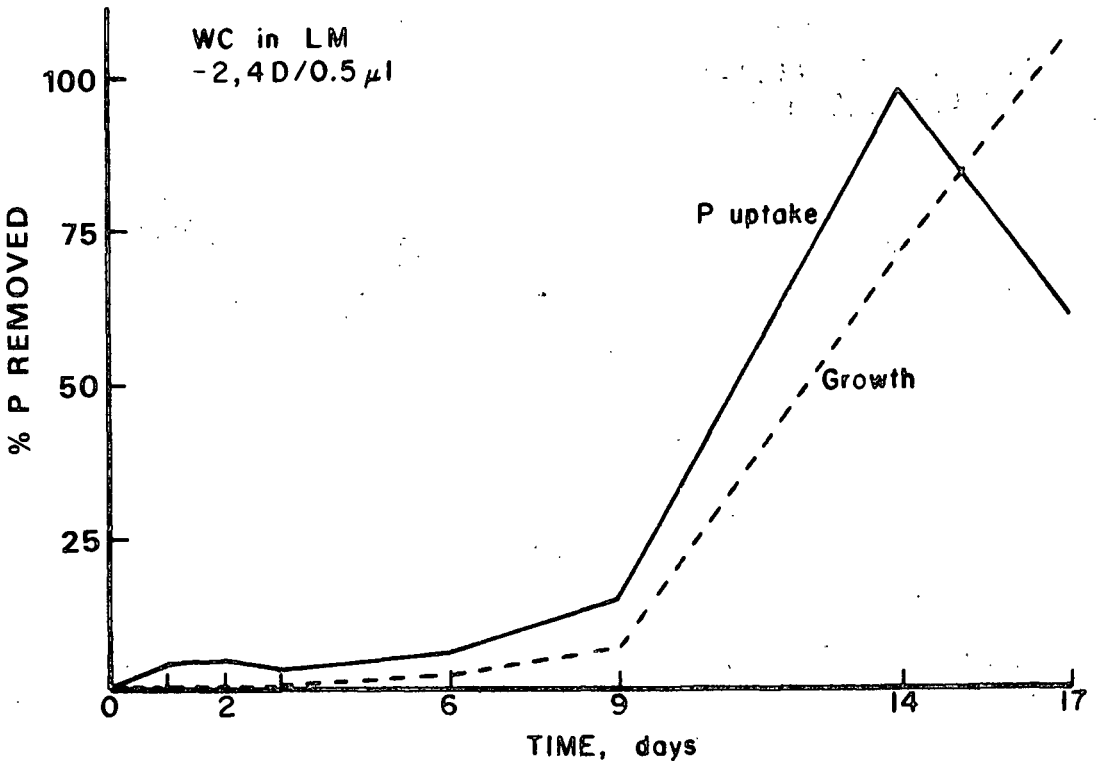


Figure 12. Phosphate uptake for wild carrot cells in LM medium without 2,4-D.

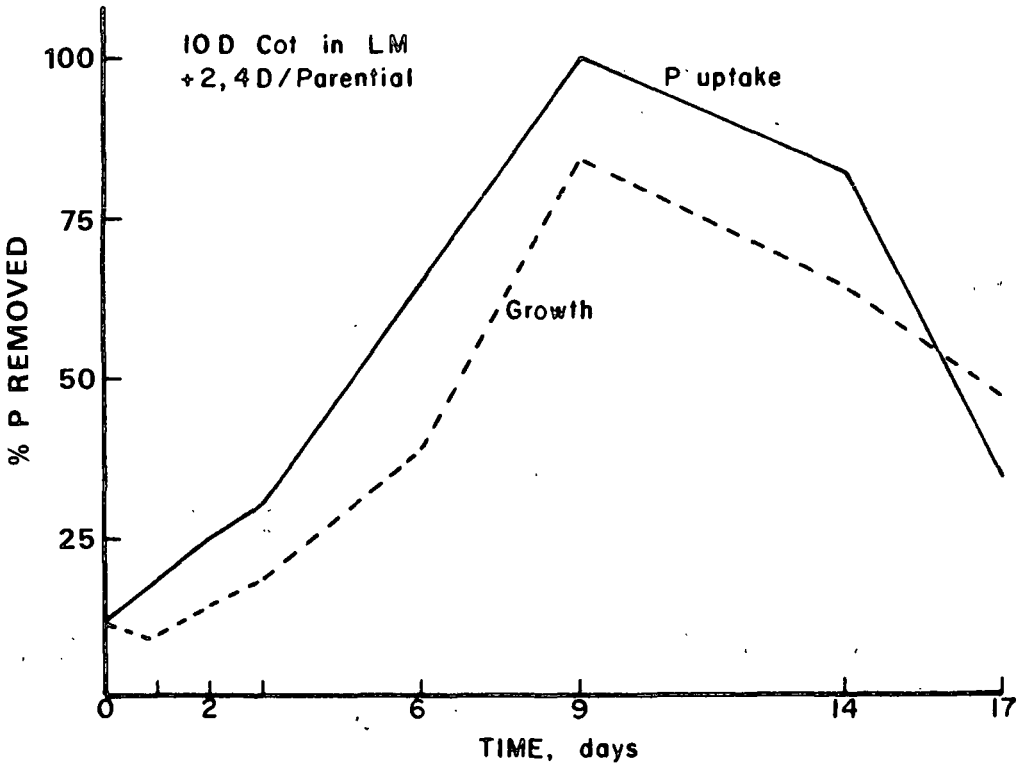


Figure 13. Phosphate uptake for loblolly pine 10-D Cot cells in LM with 2,4-D - parental inoculum.

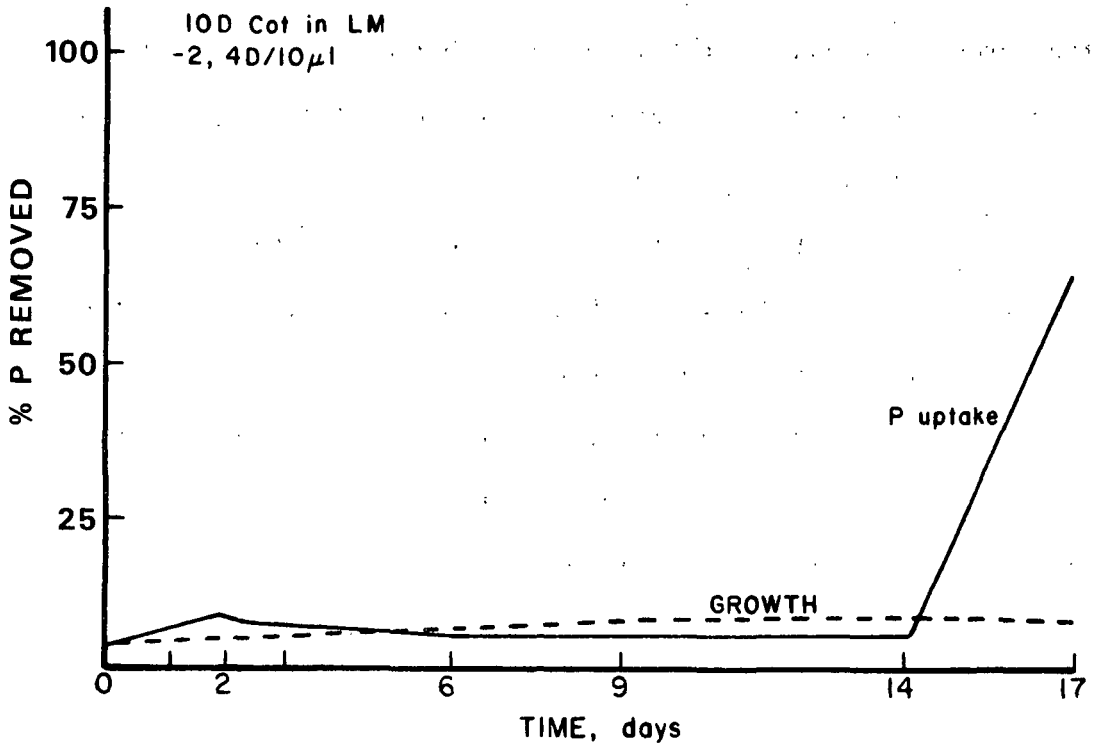


Figure 14. Phosphate uptake for loblolly pine 10-D Cot cells in LM with 2,4-D - 10 μL inoculum.

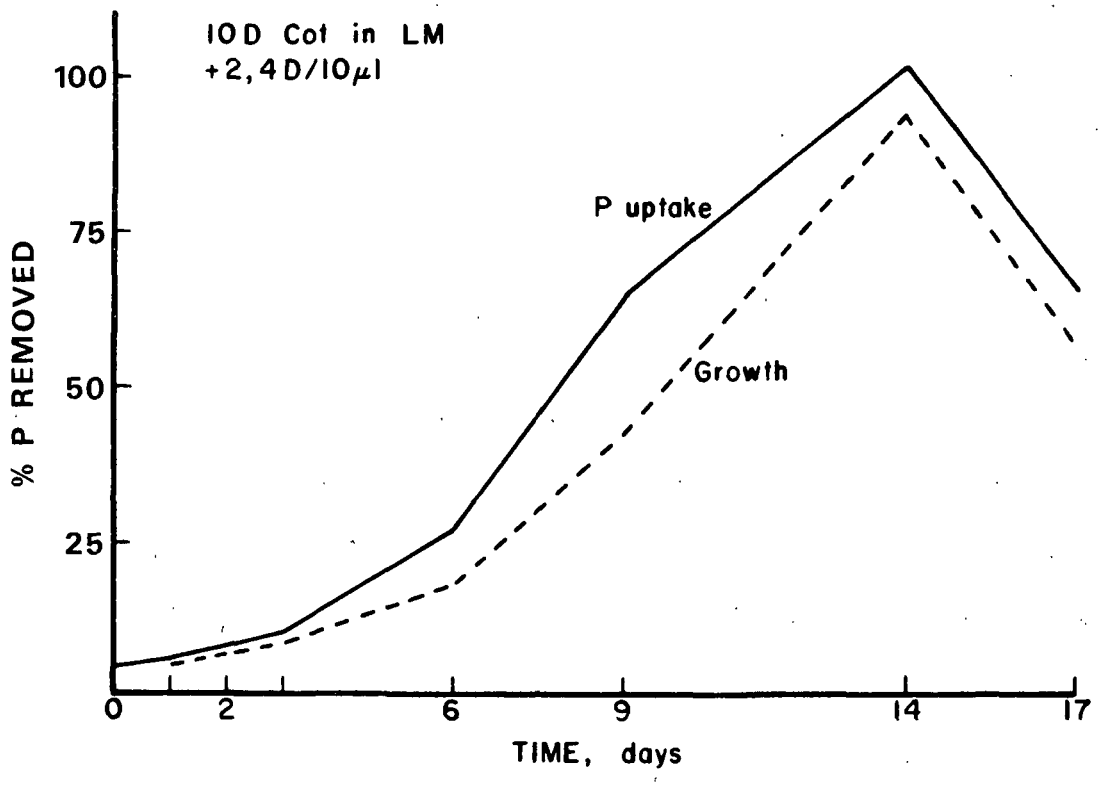


Figure 15. Phosphate uptake for loblolly pine 10-D Cot cells in LM, minus growth regulator.

culture at the parental inoculum density, which is being subcultured every 10 days, should have some endogenous phosphate left in its cells at the time of subculture. This becomes important when examining Fig. 13. When trying to obtain embryogenesis by removing the growth regulator, no growth occurs. The question then becomes, is there no growth because removal of the growth regulator blocks phosphate uptake and hence no energy production? Because there appears to be endogenous phosphate in the cells which are being placed into the minus growth regulator medium, and also because there appears to be some initial phosphate uptake by these cells (Fig. 15), if there is a problem with energy production in these cells, it is probably not due to the availability of phosphate. One possibility could be in the conversion of phosphate to ATP.

In order to answer some of these questions more fully, additional studies will be conducted on ATP generation by our cells.

EFFECT OF LOWERING CALCIUM OR INCREASING PHOSPHATE IN LM ON MAINTENANCE OF THE 10-D COT LOBLOLLY LINE

Introduction

Previous media development experiments have shown phosphate to be one of the elements of primary importance in the medium (higher levels being better for growth). Also the extremely low levels of Ca in the LM medium have resulted in questions concerning how low a level of calcium can our cells withstand. The following experiment was undertaken to evaluate the effect of lowering calcium and increasing phosphate on growth of loblolly pine cell line 10-D.

Methods

One of our gymnosperm lines (dark grown, 10-D Cot, loblolly) was subcultured into duplicate flasks containing LM medium plus 0.5 ppm 2,4-D with the

following changes with respect to P or Ca (1 x Ca, 1/2 x Ca, 1/4 Ca, 1 x P, 2 x P, 4 x P, 8 x P, where the number expresses the concentration of Ca or P with respect to the level found in LM).

After six to eight subcultures covering a period of 3 to 6 months, the growth of the cells was evaluated.

Discussion and Conclusions

10-D Cot cells grew equally well at all Ca concentrations tested. Full strength LM contains 22 ppm Ca; thus 1/4 strength Ca would be LM with 5.4 ppm Ca. Thus the lower limit of Ca tolerated by cells is below 5.4 ppm. To determine the lowest Ca level the 10-D Cot cell line will tolerate will require the initiation of another study.

10-D Cot cells could not be maintained in any of the media containing 4 x P or greater; however, the cells did grow as well as the controls in the 2 x level of phosphate. Thus, doubling the phosphate level in our cultures was neither inhibitory nor stimulatory, indicating that there does not seem to be any need to adjust the medium with respect to phosphate level.

INITIATION OF GYMNOSPERM CELL LINES
(Explant Source, Immature Embryos)

Introduction

With respect to somatic embryogenesis, major breakthroughs have been obtained with such species as corn and grasses through the use of immature embryos as the explant source. Once embryogenesis was obtained with this source, the tissue culture system was applied to other explant sources for further trial and optimization. Many researchers feel that the younger the

explant (and tissue), the easier it is to establish somatic embryogenesis in a species.

Following these lines of reasoning, studies were conducted using excised immature embryos from loblolly pine as a tissue source for initiating new cell suspension lines. This was the first time this laboratory has used this material as the explant source for initiating cell lines.

Methods

Immature embryos representing various stages of development were placed on agar containing thirty different treatments (3 media x 10 growth regulators, Table X).

TABLE X

TREATMENTS USED IN INITIATING CELL LINES FROM LOBLOLLY PINE IMMATURE EMBRYOS (SUMMER, 1983)

Growth Regulator	Medium		
	LM	#10[a]	#11[a]
None			
0.5 2,4-D			
2.5 2,4-D			
0.5 2,4-D, 0.1 BAP	The above media were used with each of the growth regulator(s) indicated at the left.		
2.5 2,4-D, 0.1 BAP			
0.5 2,4-D, 0.5 BAP			
2.5 2,4-D, 0.5 BAP			
2.5 2,4-D, 0.1 ABA			
2.5 2,4-D, 0.1 GA4-7			
5 NOAA, 0.1 BAP			

[a]These media are from previous wild carrot somatic embryo studies and are the media treatment numbers from those experiments.

Once a callus was established, part of the callus was placed into liquid medium to render a liquid cell suspension.

Results

The total number of embryos placed on agar (100%) vs. the total percent of embryos transferred and the percent of embryos yielding liquid cell lines are shown in Fig. 16.

Discussion and Conclusions

Figure 16 may be used to show that of the 3000 immature embryos placed on the 30 different media treatments representing 9 different collection points (or stages of development) we obtained no liquid cell suspensions originating from the prelaunch stages of embryo development. Second, the overall efficiency of obtaining lines was low. In all cases the efficiency was less than 25%. Thus, we must improve methods of obtaining lines from the earlier stages of development, and we must obtain a higher establishment efficiency rate for lines from this explant source.

Further work with these cell lines will center around (1) trying to induce somatic embryogenesis, (2) screening cell lines or colonies from them using biochemical selection techniques, and (3) monitoring some of the lines using the biochemical markers we have developed.

POLYAMINES AND ENZYME LEVELS IN LOBLOLLY PINE SUSPENSION CULTURES

Introduction

Our studies have shown that elevations of polyamine levels and their biosynthetic enzymes play a major role and may be essential for wild carrot embryogenesis. Increased polyamine levels, especially spermidine, are also associated with natural embryo development in pine seeds. It is reasonable to assume, therefore, that conifer cultures should exhibit rises in polyamine

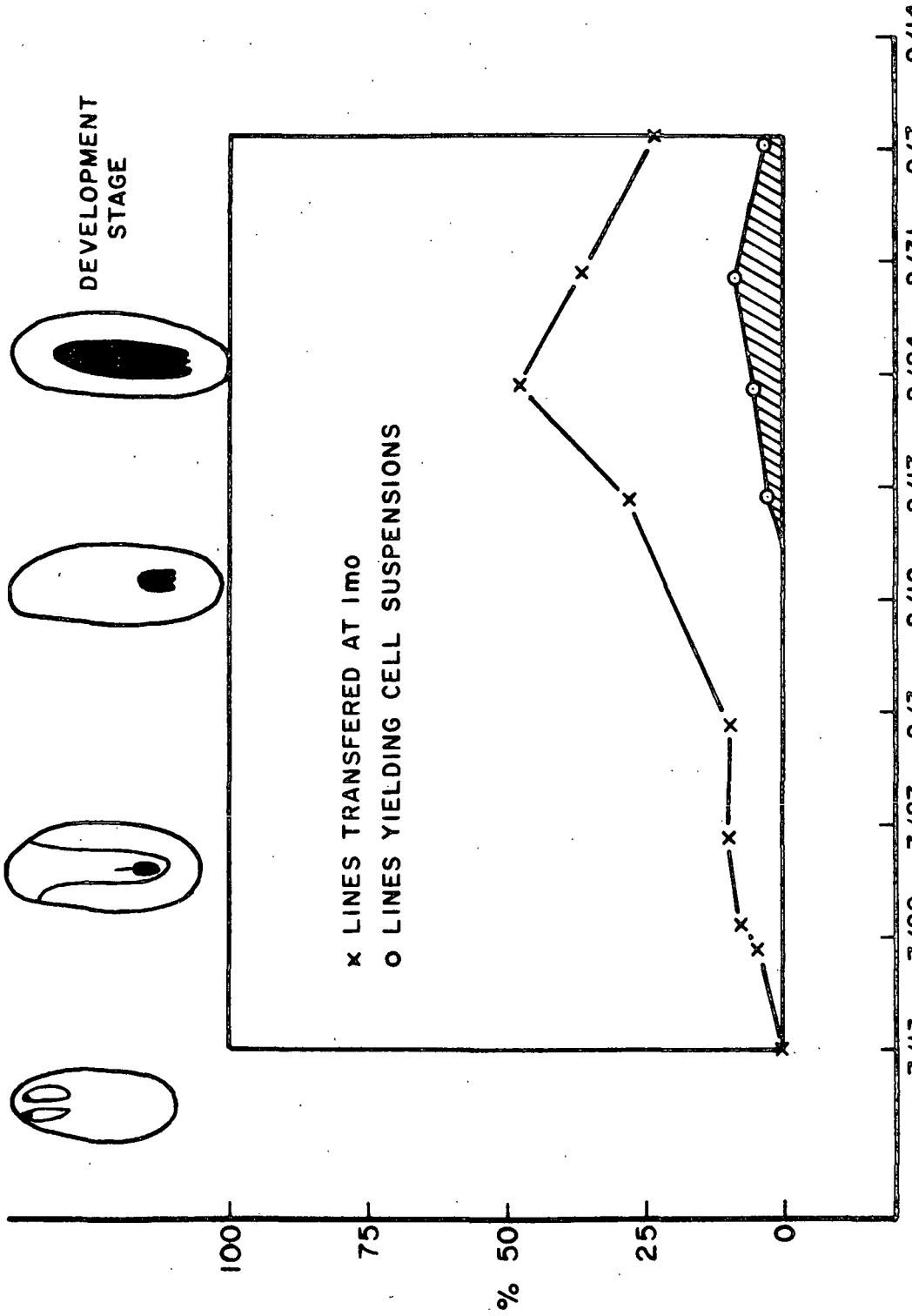


Figure 16. Percent lines successfully obtained from loblolly pine immature embryos.

levels when induced to undergo embryogenesis. This study was designed to determine how polyamines and the enzymes ADC and ODC are affected when loblolly pine suspension cultures are subjected to a standard embryogenesis induction protocol.

Methods

A suspension culture of loblolly pine was used in a procedure similar to that routinely used to induce wild carrot embryogenesis. After being screened and washed, the density of the cells was adjusted (10 µL packed cells/mL medium). The cells were suspended in fresh medium and both + and - 2,4-D treatments were established. Cell samples were collected from the flasks at the appropriate times and stored for later polyamine analyses. Enzyme assays were performed on the day of cell collection. All analyses were carried out as described elsewhere in this report, except that the enzyme assays were carried out at pH = 7.5.

Results

Preliminary results (enzyme data) of this study were presented in Report Ten (1983) and appear in Fig. 17. No significant elevations in ADC or ODC were noted in either + or - growth regulator treatments. The absolute activities of the enzymes were also lower than those in wild carrot cultures.

Polyamine levels did rise during the early sample points in both the + and - 2,4-D cultures. Putrescine exhibited significant elevations in the - 2,4-D treatment, although it did not reach levels observed in carrot tissues. Spermidine levels also rose in the tissues (Fig. 18).

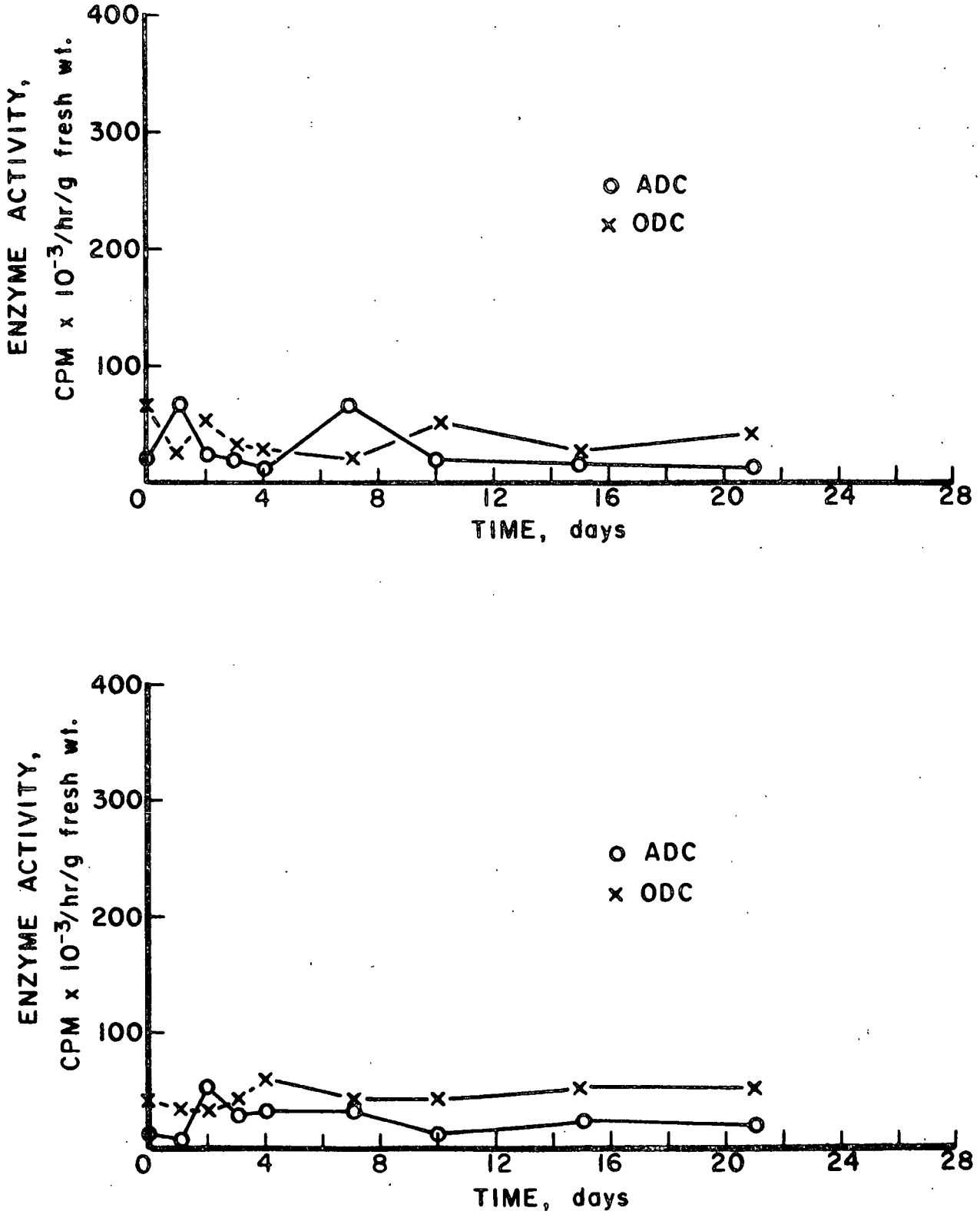


Figure 17. ADC and ODC enzyme activities of loblolly pine cells grown with 2,4-D present (top) and absent (bottom).

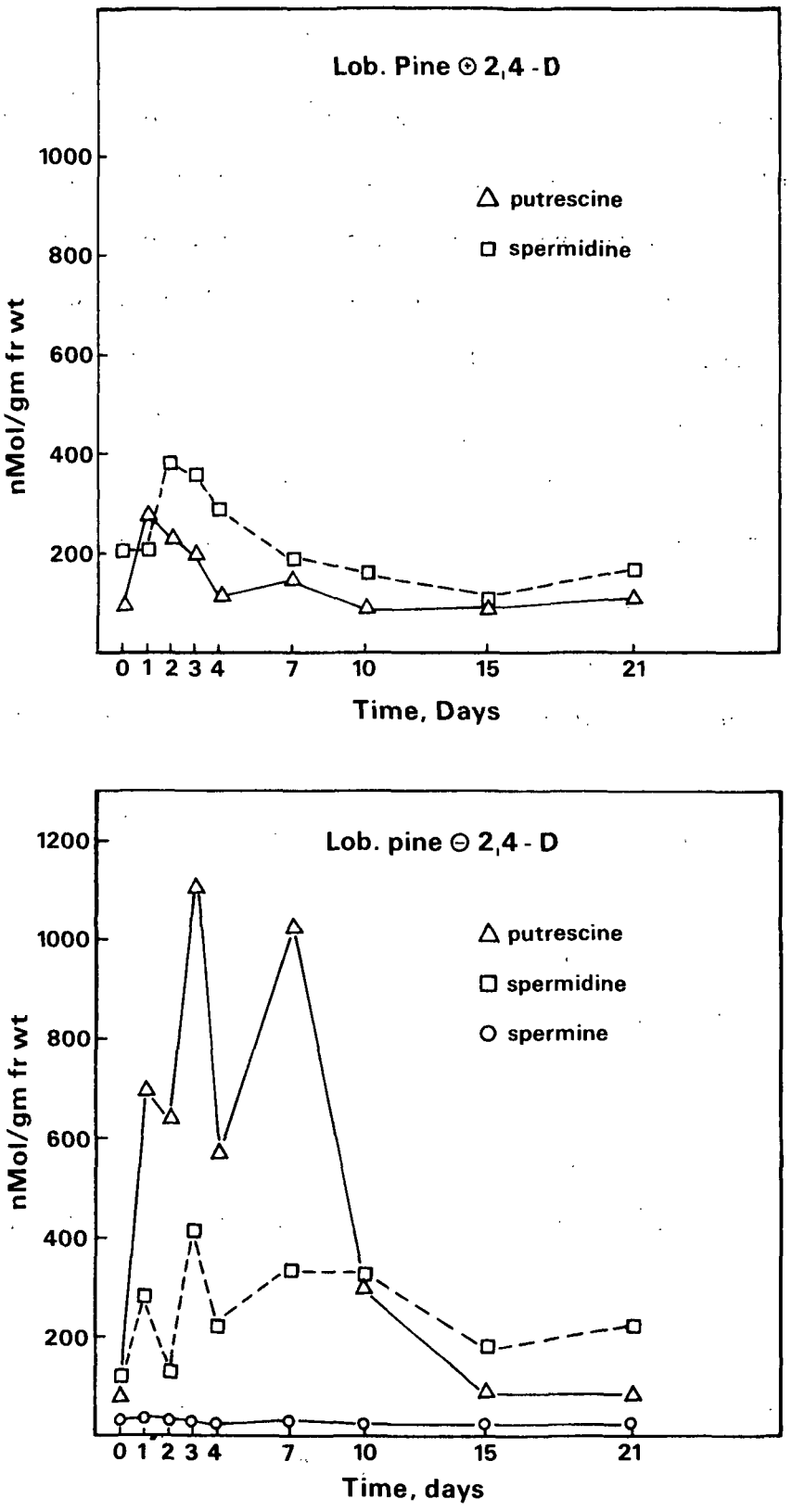


Figure 18. Free polyamine levels in loblolly pine suspension cultures.

Conclusions

When exposed to embryo inducing conditions, the loblolly suspension cells did respond by exhibiting elevated levels of putrescine and smaller elevations of spermidine. Putrescine did not increase to the levels seen during wild carrot embryogenesis, however, and the putrescine levels remained elevated longer than in embryogenic carrot cultures. Putrescine levels also remained elevated for a longer period in carrot cultures not undergoing embryogenesis (+ 2,4-D cultures, see Report Ten, 1983). It also appears that levels of spermidine, the polyamine very likely having an important role in plant embryogenesis, never rose high enough to become the dominant polyamine as in natural pine embryogenesis. In relation to our model systems (carrot, natural pine), we expected the putrescine levels to rise to higher levels and to reach a better defined peak earlier during the subculture period. Spermidine would also be expected to reach higher concentrations, with a well-defined peak occuring between days 3 to 9 of culture growth. It appears that the inability of the loblolly pine cultures to form embryos or other organized structures is reflected, or at least partially caused, by deficiencies/blocks of polyamine metabolism.

An interesting observation from this study involves the inconsistency of the enzyme data and the polyamine levels in the minus 2,4-D treatment. Although putrescine levels rise markedly, the activities of the putrescine biosynthetic enzymes, ADC or ODC, do not increase. This problem can be reconciled by a number of hypotheses, the first being that the putrescine is not enzymatically synthesized during the early period of culture growth; rather it is released from a storage or conjugated form. Polyamine-phenolic conjugates have been reported in a number of plant systems (16). Another possible cause of

the low levels of enzyme activity observed in the cells would be the presence of enzyme inhibitors released upon homogenization of the cells during the enzyme assay. These inhibitors would not affect *in vivo* enzyme activity, but would result in erroneously low *in vitro* enzyme activity measurements.

INFLUENCE OF LOBLOLLY PINE EXTRACTS ON WILD CARROT ADC ACTIVITY

Introduction

As described in previous studies, the measured activities of both ADC and ODC in cultured loblolly pine cells have been relatively low. One possible cause of this would be the presence of enzyme inhibitors of ADC/ODC which are released upon homogenization of the cells during the enzyme assay. This study was designed to test for the presence of enzyme inhibitors in loblolly pine cell homogenates.

Methods

Loblolly pine suspension grown cells were homogenized in extraction buffer as described earlier. Measured amounts of this homogenate were then added to wild carrot cell homogenates which were known to contain elevated enzyme levels. The effects of the pine cell extract upon the wild carrot enzyme measurements were then determined. The effects of the pine homogenate upon measured carrot enzyme activity due to dilution of the assay system was calculated so that expected enzyme activity levels could be predicted. Therefore, if the measured enzyme levels were lower than predicted values, the presence of an enzyme inhibitor would be suspected in the pine homogenate.

Results

Table XI contains values for measured enzyme levels in loblolly pine and wild carrot homogenates (controls), the observed enzyme levels in the test

system containing both pine and carrot extracts, and predicted values for the pine/carrot mixture (in the last line). The measured enzyme activities in the homogenate mixture treatment were not significantly lower than the calculated, predicted values.

TABLE XI

EFFECT OF LOBLOLLY PINE EXTRACT ON WILD CARROT ENZYME ACTIVITY

	Enzyme activity, nMol CO_2/g hr	
	ADC Activity	ODC Activity
Loblolly Pine	0.8 ± 0.1	0.2 ± 0
Wild Carrot	9.2 ± 3.8	0.4 ± 0.1
LP + WC	5.5 ± 0.3	0.3 ± 0
(predicted)	(5.0)	(0.3)

Conclusions

This experiment, although only carried out one time with one line of loblolly pine and wild carrot each, strongly suggests that an inhibitor of ADC or ODC is not present in homogenates of cultured loblolly pine cells. These results, along with the results of previous experiments, suggest that polyamines may be derived from a source other than (or in addition to) enzymatic biosynthesis in cultured loblolly pine.

INFLUENCE OF POLYAMINE PRECURSORS ON CULTURED LOBLOLLY PINE POLYAMINE LEVELS

Introduction

In an attempt to induce developmental processes in cultured pine tissues we think that it may be important to increase polyamine levels in the cells. This might be done by inducing biosynthetic enzyme activity in the cells, adding the polyamines to the culture medium, or providing substrates for polyamine biosynthesis. The effects of several compounds known to be utilized in polyamine biosynthesis on cultured pine tissue polyamine levels were determined in this study. This approach is valid, since it has already been determined that our cells may have low levels of a very important substrate for polyamine synthesis (arginine).

Methods

Compounds known to be precursors of polyamine biosynthesis were added to the medium of loblolly pine suspension cultures. After 6 days of growth in darkness the cells were collected and stored for later polyamine analysis. Polyamines were quantitated as previously described. The values represent the mean and S.D. of quadruplicate determinations. Values were judged significantly different from the control if $p < 0.05$.

Results

Although spermidine levels were not significantly affected by additions of any of the compounds, both arginine and agmatine did raise putrescine levels in the cells (Fig. 19). Spermine levels were also unaffected (data not shown). It should be noted that agmatine resulted in a larger elevation of putrescine than did arginine, and the agmatine was added at only 1/5 the concentration of the arginine (1 mM vs. 5 mM).

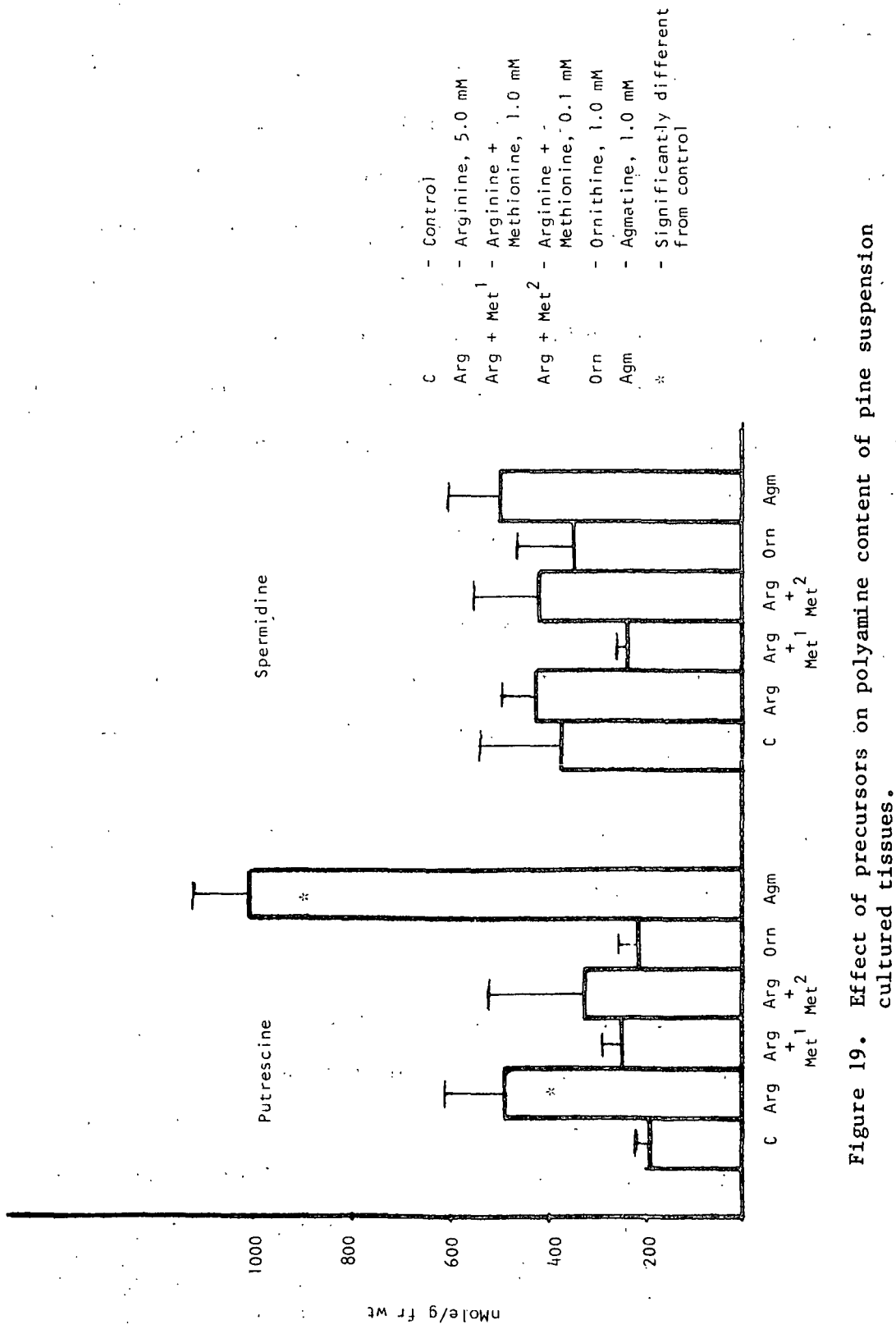


Figure 19. Effect of precursors on polyamine content of pine suspension cultured tissues.

Conclusions

It is evident from this study that putrescine levels can be affected in cultured pine cells by additions of agmatine or arginine to the culture medium. It is important to note, however, that levels of spermidine, the polyamine suspected to play a role in plant embryogenesis, were unaffected by any substrate additions. Although agmatine may be added to the medium for pine cultures in our lab, we will continue our attempts to raise spermidine levels through other means.

OBJECTIVE II RESEARCH

TANNIN CONTENT AND PAL LEVELS IN LOBLOLLY PINE CELL SUSPENSIONS

Introduction

As discussed at considerable length in the last report, conifer cell lines respond to transfer to 2,4-D-free media by producing tannins (proanthocyanidins) and failing to grow. Some further light is shed on this process in this report, particularly with regard to the role of PAL (phenylalanine ammonia lyase). Since this enzyme (PAL) is situated at a crucial junction of metabolism leading to the production of phenolic compounds (Fig. 20), it was necessary to investigate its behavior relative to tannin production by conifer cell suspensions. This happens to be a rare instance in which some of the corresponding data for the wild carrot model system is not yet in hand, but it should be obtained shortly.

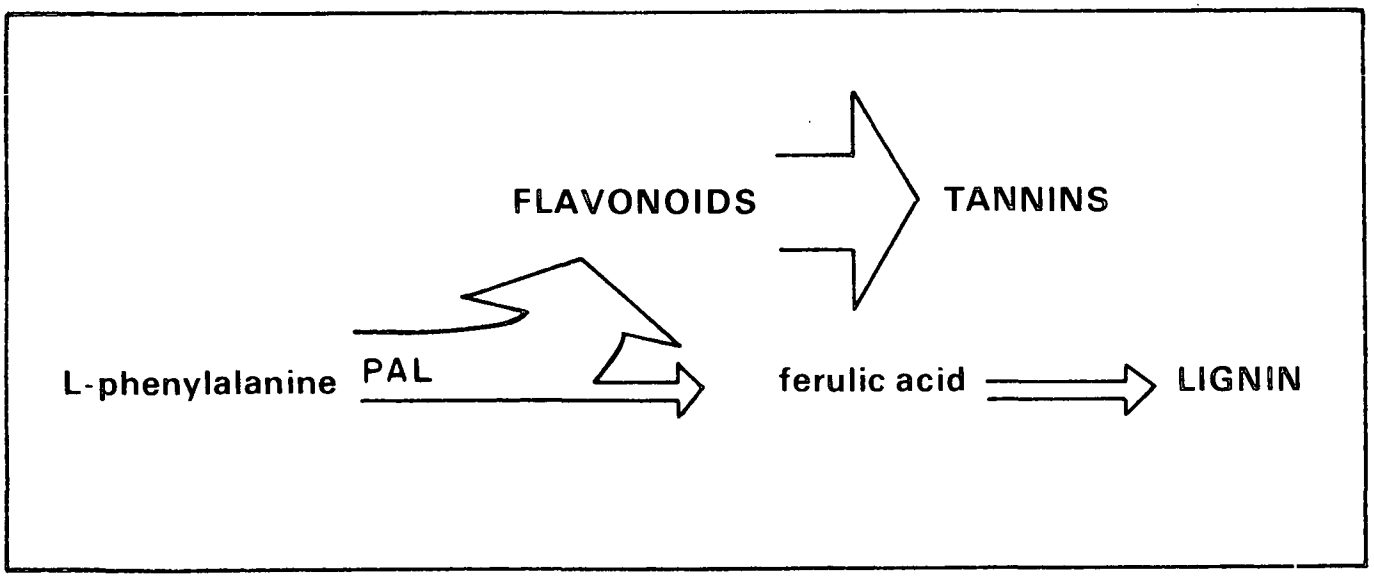


Figure 20. The metabolic position of PAL relative to the biosyntheses of tannin and lignin in cultured conifer cells.

Materials and Methods

The assay of proanthocyanidins was as given in Progress Report Ten except that in these experiments the total cell sample was analyzed rather than a cell extract. The PAL enzyme was extracted from acetone powders with 0.1M borate buffer, pH 8.8, and assayed by change in absorbance at 290 nm (17). Protein was determined by the Bradford dye procedure (18).

Results

In the data of Progress Report Ten, there were several indications that tannin synthesis by conifer suspension cells was under the control of 2,4-D. More recent data of this type are presented in Fig. 21, which shows a dramatic example of how our main loblolly pine cell line (10-D Cot) can respond to transfer to 2,4-D-free medium by producing tannin rather than embryos. Nevertheless, in the series of four large experiments with 10-D Cot which were run subsequent to obtaining the data of Fig. 21, this response was quite variable in terms of the quantity of tannin produced. The major difference relative to the data of Fig. 21 would appear to be that the stock culture for the data of Fig. 21 had been maintained at 0.1 mg/L 2,4-D rather than 0.5 mg/L 2,4-D.

The series of four experiments just mentioned were designed to examine the relationship between PAL activity and tannin production and also to find out if inhibition of PAL was a feasible route to the control of tannin production by conifer cell suspensions in 2,4-D-free media. These four experiments are labeled A, B, C, D in the order in which they were run. Experiments A, B, and C were run sequentially; experiment D followed experiment C but was initiated two months later. The same loblolly pine cell line (10-D Cot maintained on 0.5 mg/L 2,4-D) was used in all four cases; however, the inoculation density varied as

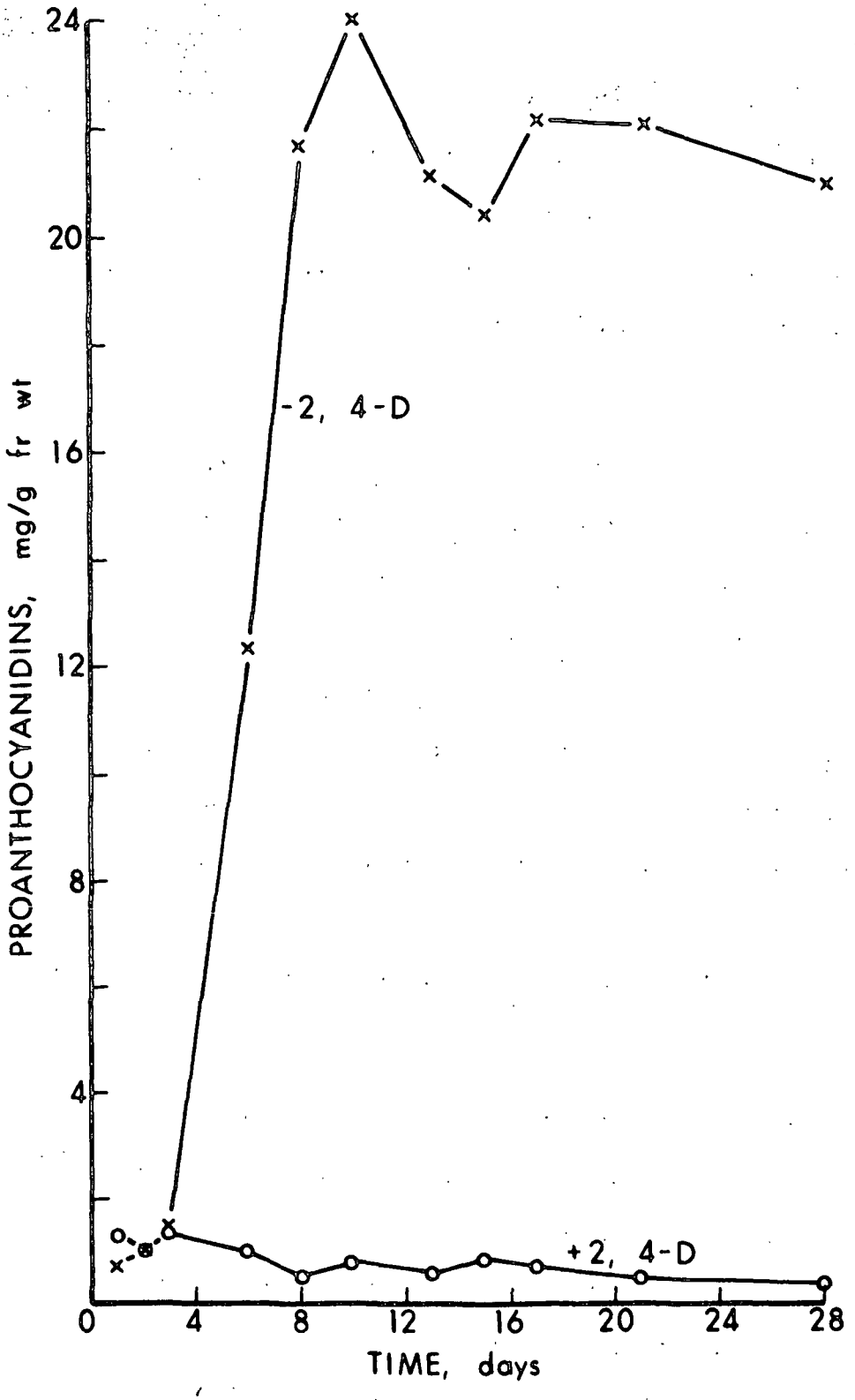


Figure 21. Tannin yield of loblolly pine main cell line 10-D Cot during proliferative (+ 2,4-D) and "embryogenic" (- 2,4-D) growth.

follows in grams/400 mL (i.e., grams/flask): A (2), B (1.4), C (1.3), and D (1.65), and the inoculum age varied as follows in days (age of stock culture): A (15), B (14), C (14) and D (12). Each experiment was monitored for in vitro PAL activity, tannin production, growth, and PAL inhibition by various compounds added in vivo. Both + and - 2,4-D systems were included in experiments A and D, whereas experiments B and C consisted only of - 2,4-D systems.

Measurements of PAL activity yielded a consistent result over all four experiments (Fig. 22 and 23). Generally, both + and - 2,4-D control cells were characterized by an early large spike of PAL activity (days 1-3), which then declined. In experiments A and D, where + 2,4-D controls were run, no PAL activity was detectable in + 2,4-D cell extracts beyond about the first week. On the other hand, in all four experiments the PAL activity in - 2,4-D control cell extracts also declined from its initial peak, but substantial activity persisted longer (3 of the 4 cases still had activity at day 13).

In contrast to the behavior of PAL, tannin production was quite variable over the four experiments (Fig. 24). No tannin was detected at any time in the + 2,4-D controls of experiments A and D. The - 2,4-D controls of experiments A and D reached maxima of 25 and 34 mg/g fr. wt., both on day 10. However, in experiments B and C the level reached was only about 4 mg/g fr. wt., around day eight, although the tannin level was considerably higher than that on day 13 in experiment B.

The growth curves that accompanied the + and - 2,4-D controls followed in the footsteps of previous experience, i.e., + 2,4-D systems grow and - 2,4-D systems do not (Fig 25). The tendency for a slight amount of growth was noted for the - 2,4-D cells in experiments A and D, but this might be attributable to

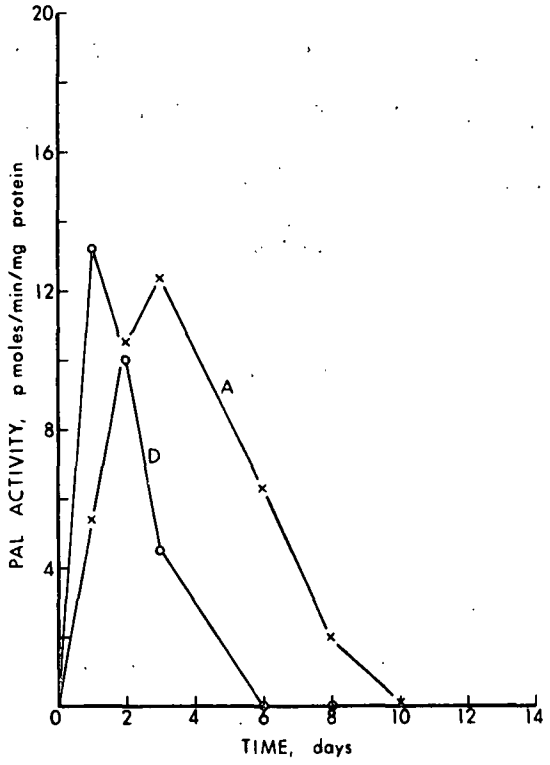


Figure 22. PAL activity as a function of time for loblolly pine cell line 10-D Cot growing in the presence of 2,4-D in experiments A and D.

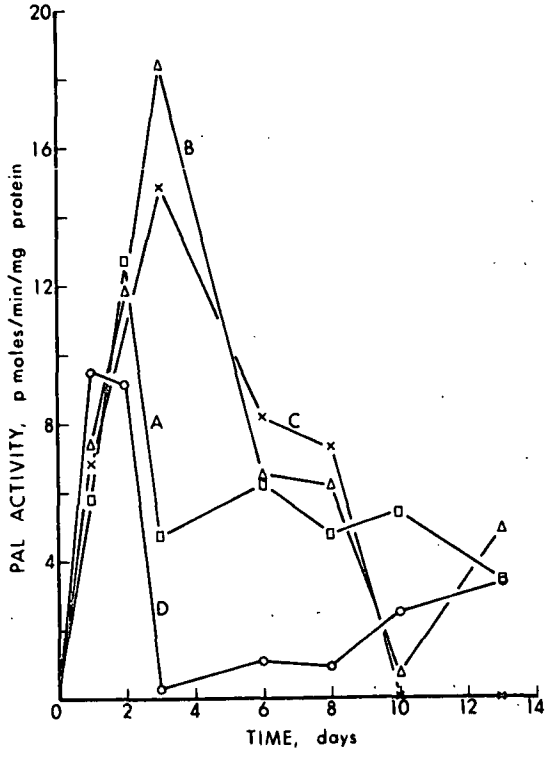


Figure 23. PAL activity as a function of time for loblolly pine cell line 10-D Cot growing in the absence of 2,4-D in experiments A, B, C, and D.

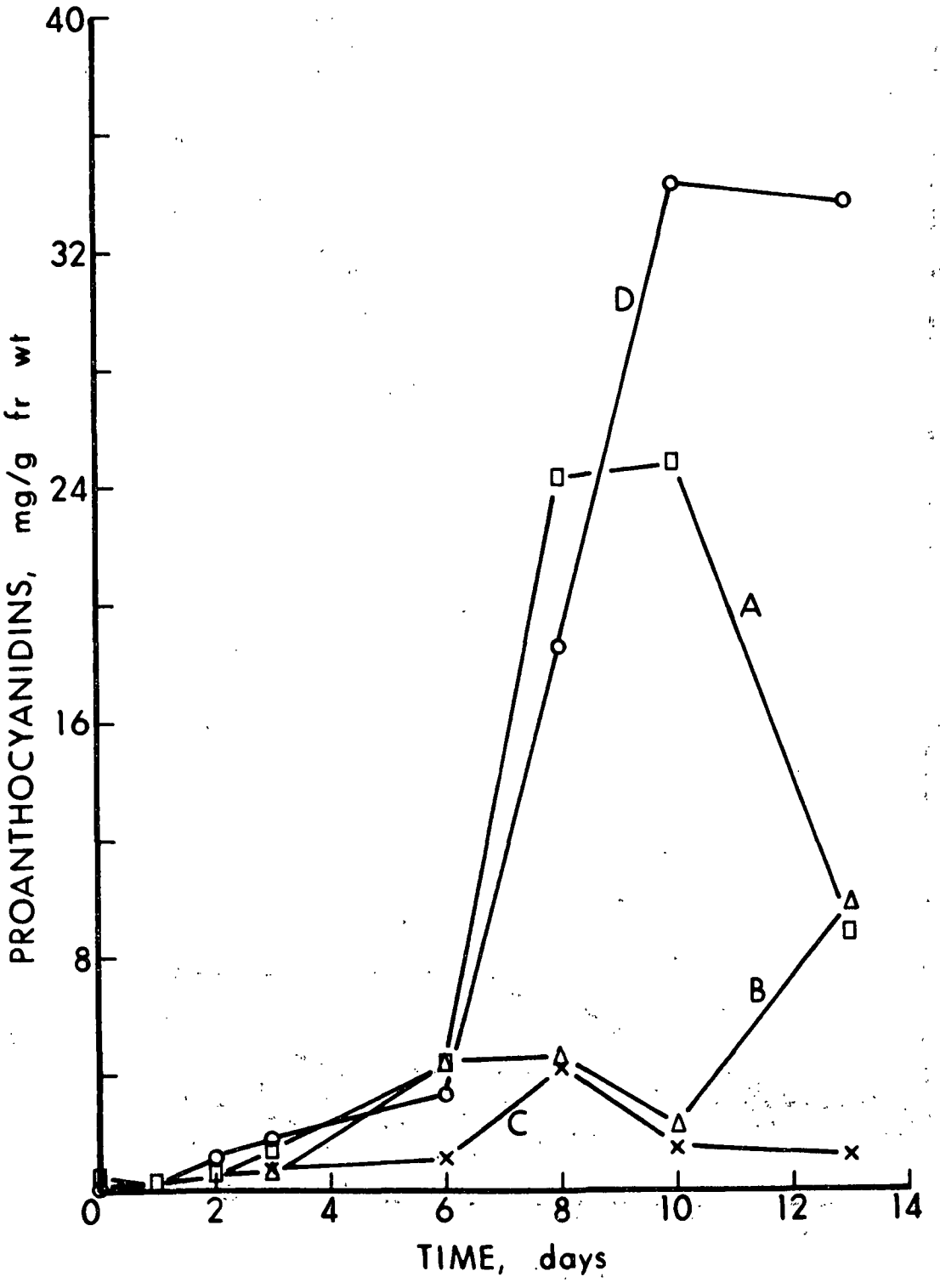


Figure 24. Tannin content as a function of time for loblolly pine cell line 10-D Cot growing in the absence of 2,4-D in experiments A, B, C, and D.

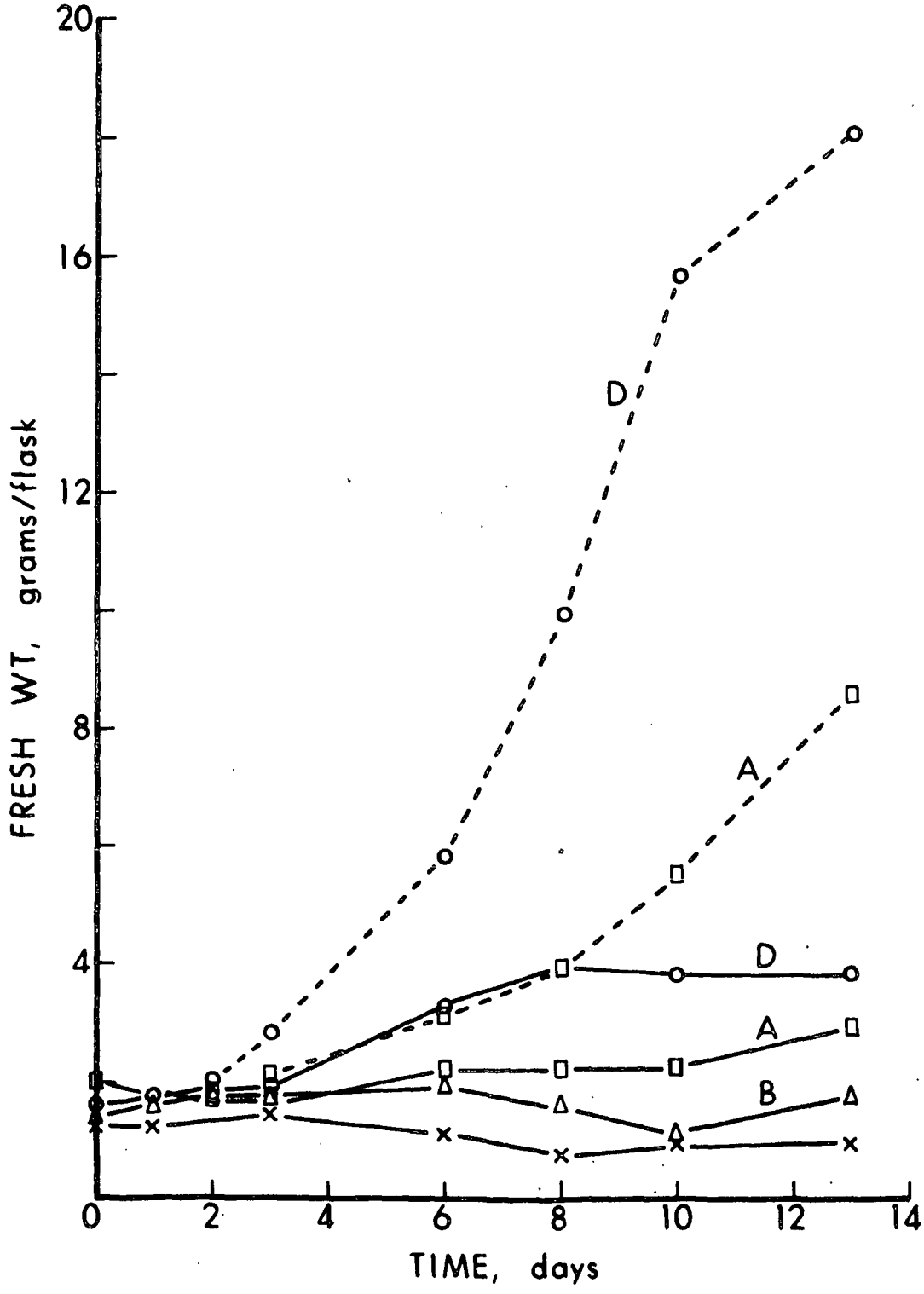


Figure 25. Growth of loblolly pine cell line 10-D Cot in the presence (experiments A and D-dotted line) and absence (experiments A, B, C, and D) of 2,4-D.

2,4-D "carryover," since these two experiments were initiated with the heavier inocula.

Compounds tested for effects on tannin production and growth as well as for their capacity to stop PAL activity induced by transfer of cells to 2,4-D-free media included hydroxylamine, o-benzylhydroxylamine (OBHA), amino-oxyacetic acid (AOA), α-aminooxy-β-phenylpropionic acid (AOPP) and cinnamic acid. Of these compounds, AOPP is the premier PAL inhibitor in the literature (19). The compounds were added to the growth media, and the concentrations used in these experiments were based upon the literature, although a "rangefinder" test has been conducted for AOPP (Fig. 26) to ascertain its compatibility with growth in the presence of 2,4-D. AOPP inhibition of PAL activity in - 2,4-D cells (experiment C) is presented in Fig. 27. AOPP lived up to its reputation, reducing the size of the PAL activity spike at day 3 by about 70%. With the other inhibitors (data not given here), the inhibition of PAL was not very effective until after the initial wave of PAL activity had passed. For example, OBHA and AOA gave complete inhibition at 10 days and later, but little before that.

Each inhibitor (at the level used) showed some capacity to block tannin production. Since the tannin yield was so variable from one experiment to the next (Fig. 24), it is not a simple matter to rank the inhibitors in this respect. For example, it can be stated that AOPP blocked essentially all of the tannin appearing in experiment C, but bear in mind that for the tannin peak in the control there was only about 4 mg/g fr. wt.; OBHA did the same in experiment A relative to a control level of 25 mg/g fr. wt. Nevertheless, it is important to note that in no case was either PAL inhibition or blocked tannin production

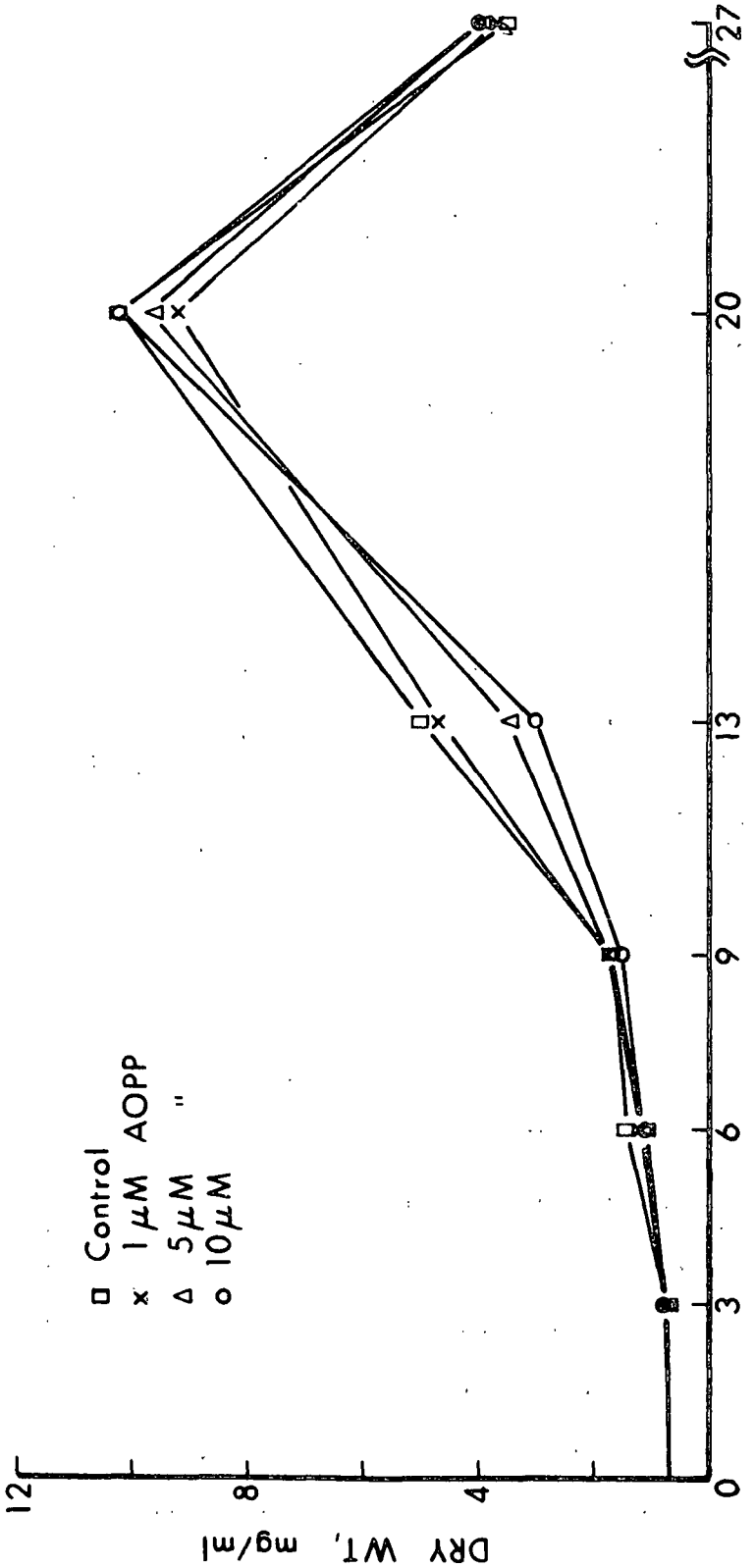


Figure 26. The effect of various concentrations of the PAL inhibitor AOPP on the growth of loblolly pine cell line 10-D Cot in the presence of 2,4-D.

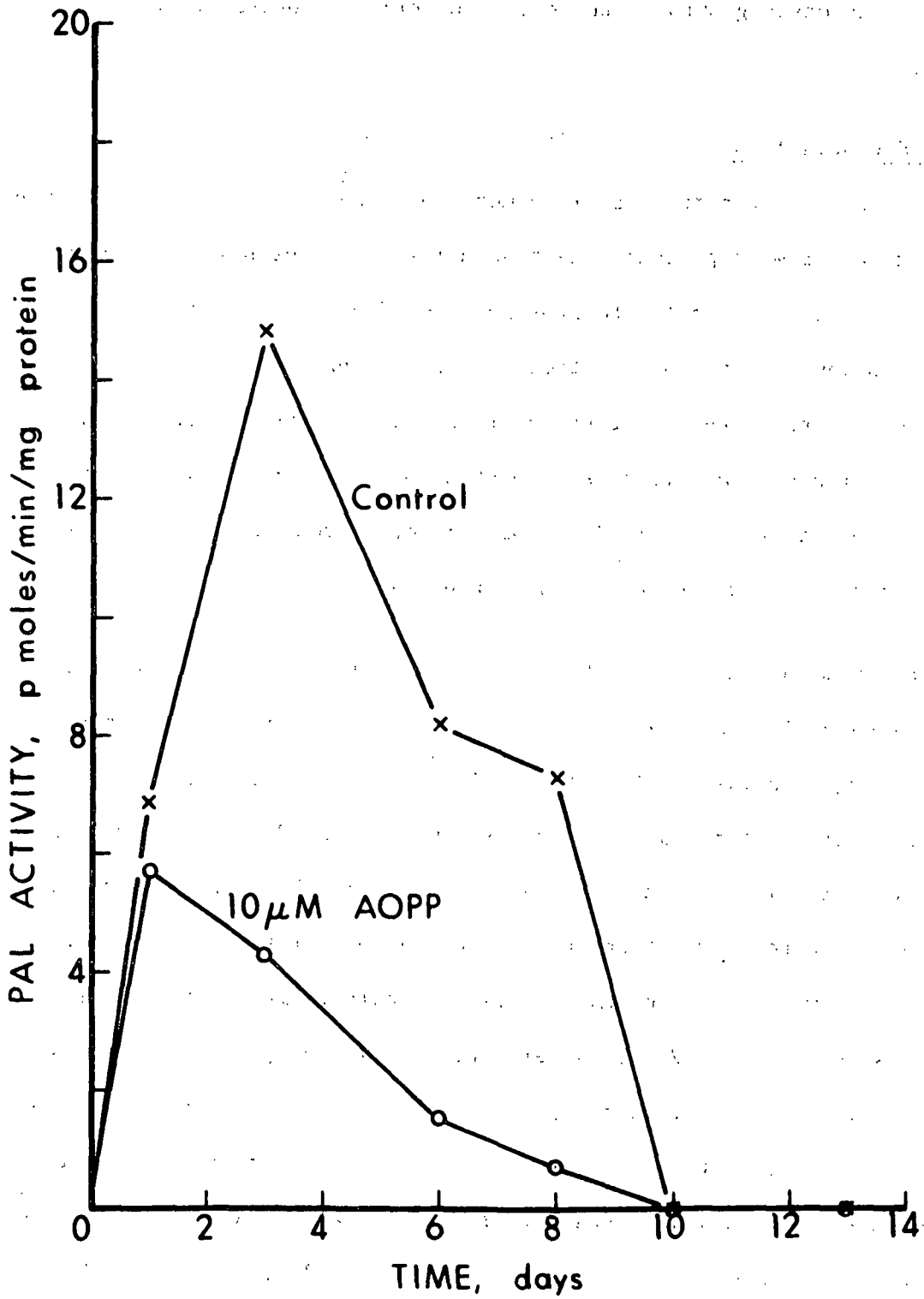


Figure 27. The effect of 10 μM AOPP on PAL activity in the absence of 2,4-D in experiment C.

accompanied by increased growth of the cells relative to control (data not presented here).

Discussion and Conclusions

Taken in toto the results given here indicate that, although it may be a contributing factor when present, the tannin itself is not solely responsible for the failure of the pine cells to grow in 2,4-D-free media. At least this would seem to follow from the observation that there were several cases where PAL inhibitors prevented the appearance of tannin but did not elicit a growth response. The strongest case in this respect is AOPP, since the data of Fig. 26 are in hand to show that the 10 μM level used in experiment C still permits substantial growth of 10-D Cot by day 13 in the presence of 2,4-D.

Another major outcome of these experiments is the absence of strong correlation between PAL activity and tannin production. Most obvious is that the early and largest spike of PAL activity is not necessarily accompanied by a burst of tannin, either immediately or after some delay. This large initial spike of PAL activity is by no means peculiar to our conifer cells; in other systems including carrot (19) it has been described principally as an effect caused by dilution with fresh medium. It is particularly interesting that + 2,4-D systems as well as - 2,4-D systems show the large spike of PAL activity soon after inoculation and yet do not produce tannins. On the other hand, the - 2,4-D systems that do accumulate tannin have a more persistent PAL activity, and this "residual" PAL activity does show some correlation with tannin accumulation. This is most easily discerned in the tannin buildup near the end of experiments B and D.

Although our own data on the PAL activity in + and - 2,4-D wild carrot model cells are not yet available, there is significant literature on + systems (19), and it has been noted in our previous reports that tannin and related anthocyanin production occur only in late development stages of carrot somatic embryogenesis. We are beginning to examine early stages of phenolic metabolism in both wild carrot and conifer cells, for it appears likely that, if phenolics can be blamed for the failure of conifer cells to grow under - 2,4-D conditions, more than tannins per se are apt to be involved. Furthermore, PAL activity per se doesn't necessarily lead to nongrowing systems, since, if 2,4-D is present, only the initial burst of PAL activity (which seems harmless, perhaps even necessary) occurs. This + 2,4-D PAL burst must make at least cinnamic acid but never tannin. What are the products that accumulate in this case? The literature suggests that hydroxycinnamic acids are likely culprits in systems which do not grow and that gibberellins may be part of the control system.

Experiment D really provides a glimpse of hope for the future in this area. Notice how these cells were growing some under - 2,4-D conditions until tannin production really took off around day 8. No PAL inhibitors were used in this experiment but had, e.g., AOPP been present the results might have been very interesting. What sets experiment D apart was not inoculum density, but the fact that the inoculum was only 12 days old (probably had not reached stationary phase).

LOBLOLLY PINE LAUNCH EXPERIMENTS

Introduction

This section consists of a summary of largely unmonitored launch experiments with old and new loblolly pine cell lines. For the most part the launch conditions tried here encompassed the various conventional manipulations used around the world by various laboratories to obtain somatic embryogenesis in other species. There are some instances where additives intended to offset known deficiencies of the conifer cells were present during launch.

Materials and Methods

The media used for these launch experiments were based on the LM formulation. Note that LM-3 differs from ordinary LM in that most of the Mg is present as nitrate instead of sulfate. As a result, nitrate levels are higher and sulfate lower; more inositol is also present in LM-3. Variations of LM are noted in conjunction with presentation of the data. In some cases the tannin content was monitored as described in the preceding section of this report. Replication of the treatments was often minimal as given in the text.

Results

For convenience, these results are presented in historical order as indicated by research plan (RP) numbers associated with the tables and figures. In RP132 the main loblolly pine cell line (10-D Cot) was launched in conventional LM-3 medium modified by all of the combinations of auxin and cytokinin in the matrices of Table XII. Putrescine, was also a variable as indicated. No organized structures were observed in any treatment. However, the experiment was run at three different inoculum densities, and some data on growth and tannin

production were collected for the combinations listed in the upper portion of Table XII. Duplicates were run, but one was used for dry weight and the other for tannin analysis. The effects of varying the inoculum density from the standard (10 µL/mL) upon dry weight and tannin yields are presented in Fig. 28 and 29, respectively. The growth responses to BAP and putrescine treatments at standard inoculum density are presented in Tables XIII and XIV; the corresponding tannin production appears in Fig. 30 and 31.

The loblolly pine cell line 10-D Cot was launched by lowering [2,4-D] at two inoculum densities in several variations of LM-3 medium in RP 140. Again, no structural organization occurred, but there were effects on fresh weight and tannin production as presented in Tables XV and XVI, respectively. Duplicates were run in RP 140 but stopped at different times; only the 11-day sample data are given. Further combinations were tested with 10-D Cot and four new cell lines in RP 142 as indicated in Table XVII. No embryogenic responses were evident in this case either, which included trials on three LM variations and additions of polyamines.

The effect of switching 10-D Cot to other auxins (NOAA and NAA) was explored as a launch strategy (Table XVIII) in RP 148 but to no avail. Tannin production (duplicate means) in response to NOAA and NAA is depicted in Fig. 32. The RP 198 launch experiment (Table XIX) was conducted in duplicate on Gelrite containing 1/2 LM. Agmatine and an extract of LP pollen were included in the test matrix. In addition to 10-D Cot and a white pine cell line, 14 new cell lines of loblolly pine were tested in this launch which also failed to yield organizational responses.

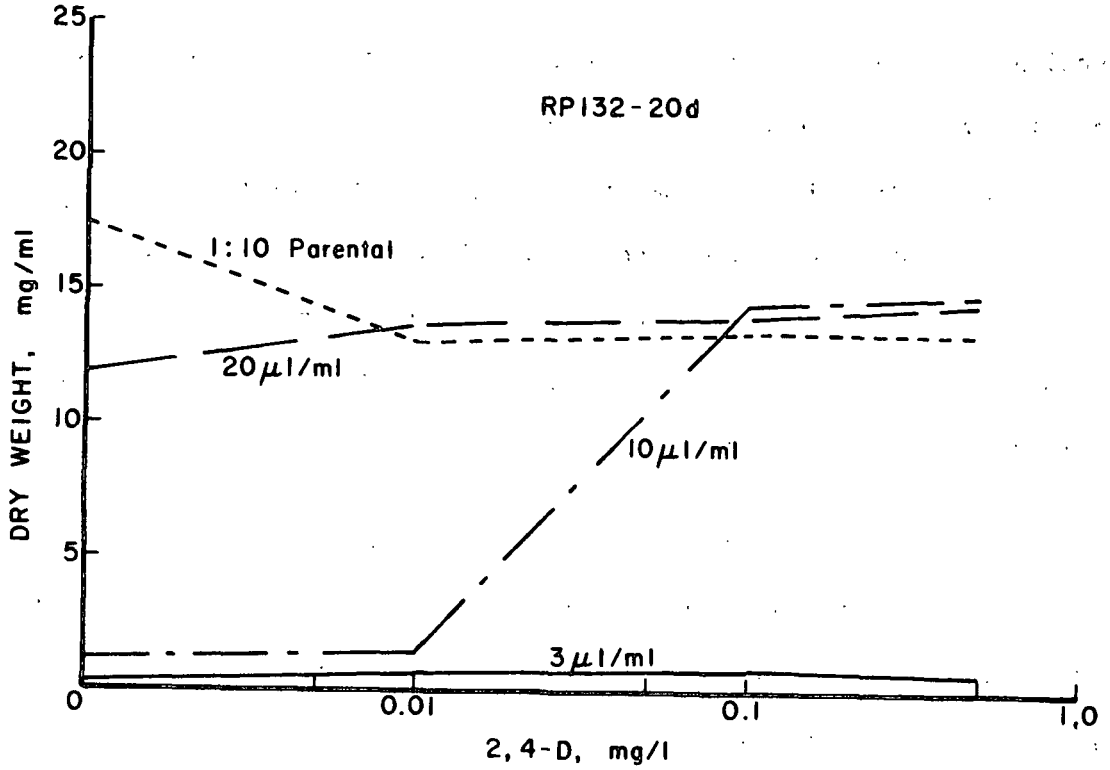


Figure 28. Growth of loblolly pine cell line 10-D Cot measured at day 20 as a function of inoculum density and 2,4-D concentration.

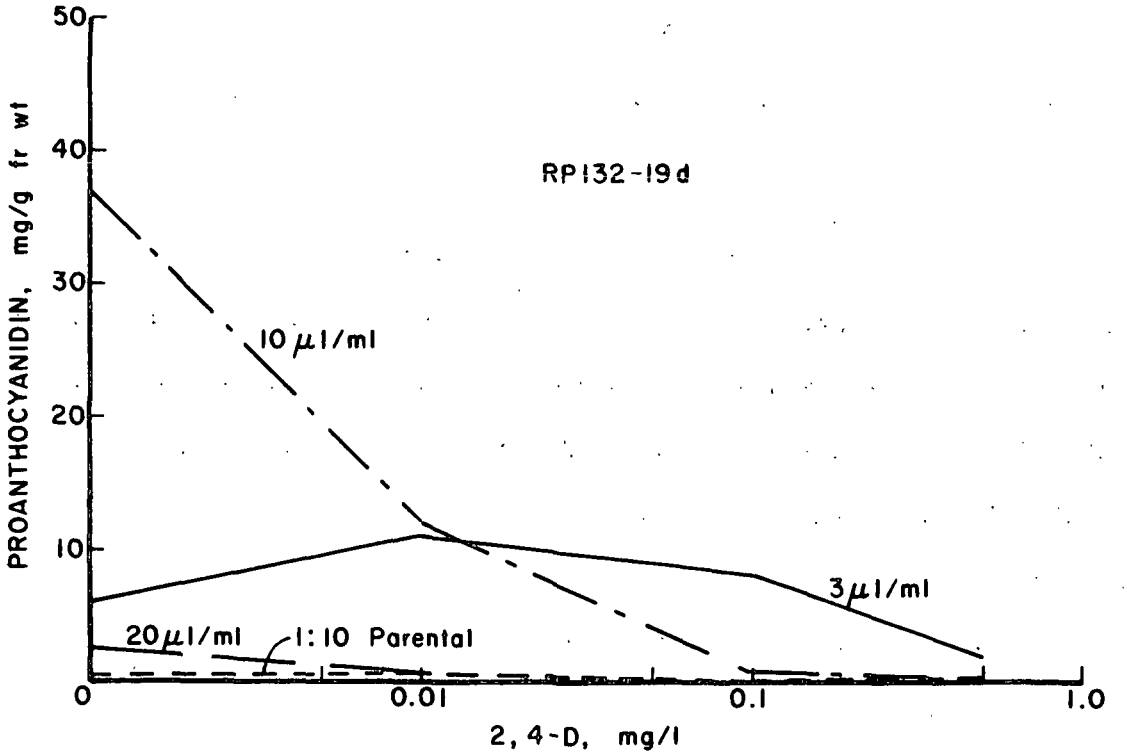


Figure 29. Tannin content of loblolly pine cell line 10-D Cot measured at day 19 as a function of inoculum density and 2,4-D concentration.

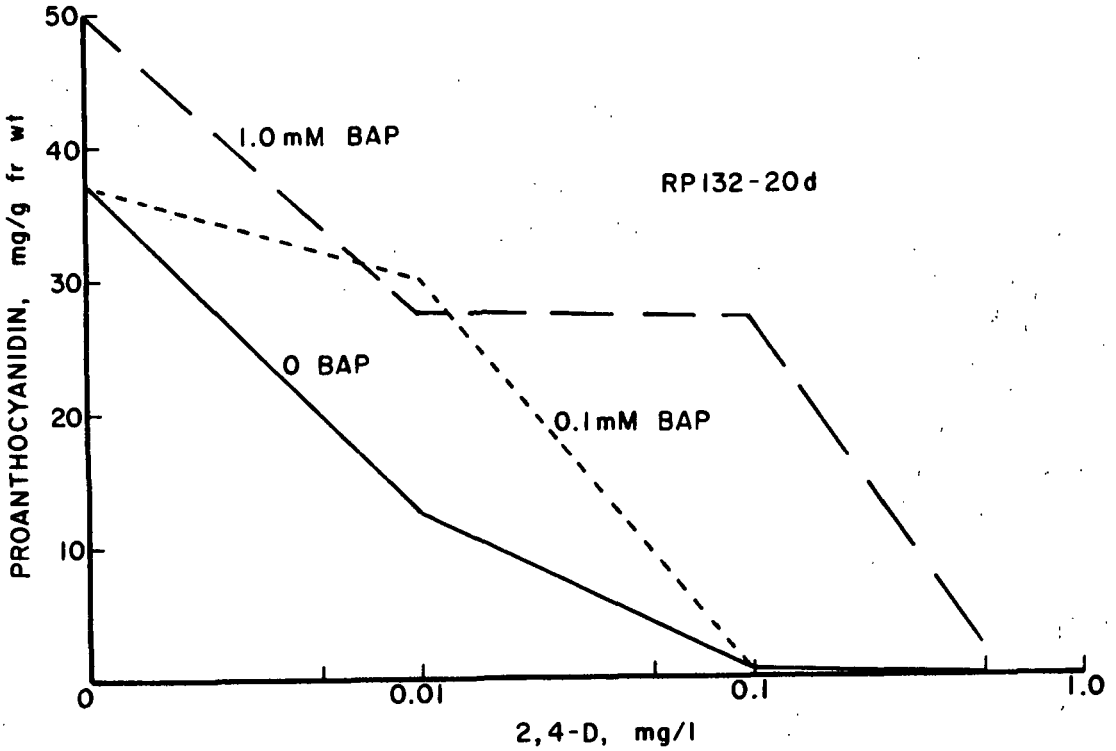


Figure 30. Tannin content of loblolly pine cell line 10-D Cot measured at day 20 as a function of BAP and 2,4-D concentrations.

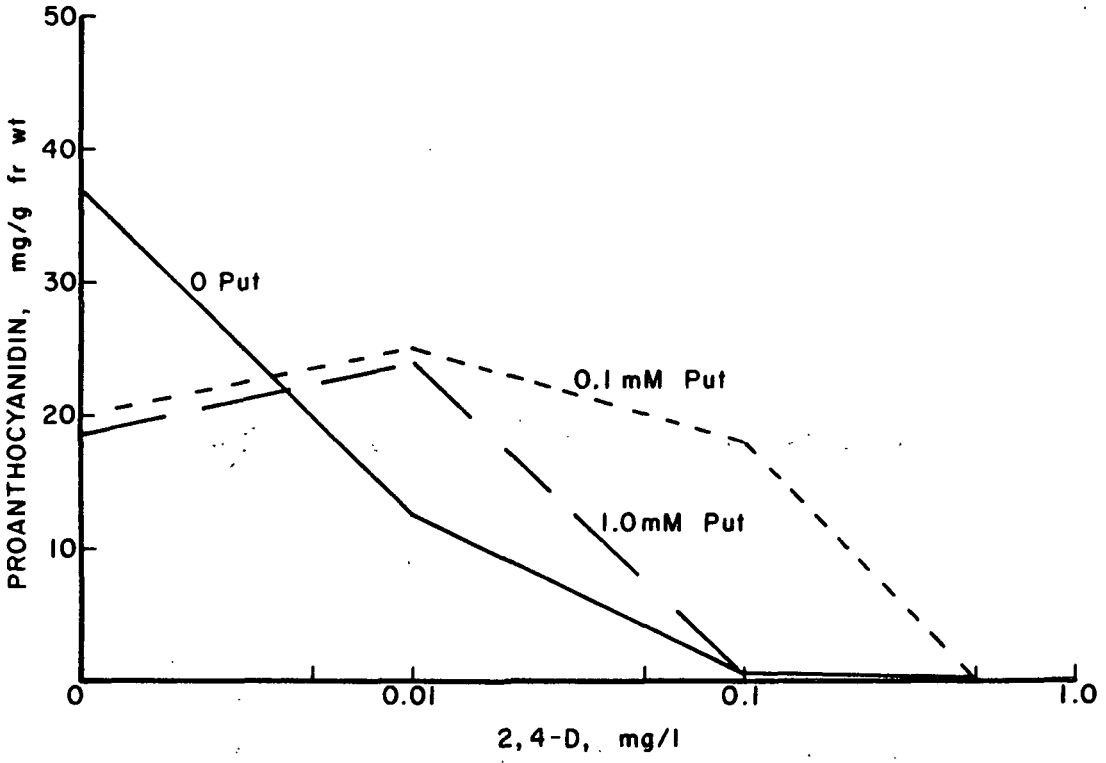


Figure 31. Tannin content of loblolly pine cell line 10-D Cot measured at day 20 as a function of putrescine and 2,4-D concentrations.

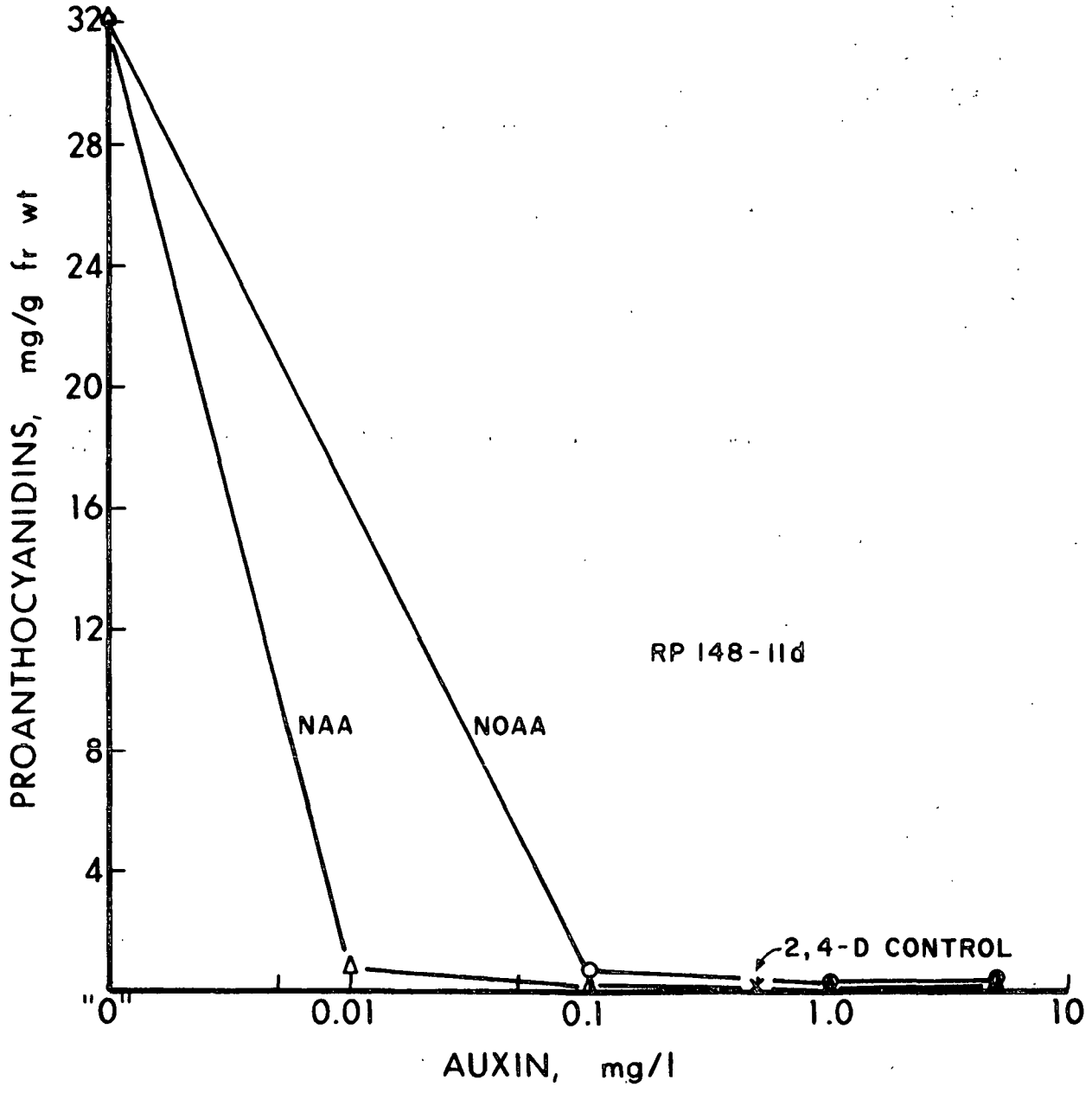


Figure 32. The effect of NOAA and NAA on the tannin content of loblolly pine cell line 10-D Cot at day 11.

TABLE XII

10-D COT LAUNCH MATRICES (RP 132)

	Additives				
	BAP, mg/L			Putrescine, mM	
2,4-D, mg/L	0	0.1	1.0	0.1	1.0
0.5	x	x	x	x	x
0.1	x	x	x	x	x
0.01	x	x	x	x	x
0	x	x	x	x	x

	BAP, mg/L			
Auxin, mg/L	0	0.1	1.0	0.1 + 0.1 mM Putrescine
2,4-D, 0.01	x	x	x	x
2,4-D, 0	-	x	-	-
NAA, 0.01	-	x	-	-

TABLE XIII

10-D COT YIELD VS. BAP ADDITION (RP 132)

	2,4-D, mg/L			
	0	0.01	0.1	0.5
BAP, mg/L	(20-Day Dry Weights, mg/mL)			
0	1.1	1.4	14.5	14.9
0.1	1.1	1.4	17.1	---
1.0	1.0	1.3	11.7	11.4

TABLE XIV

10-D COT YIELD VS. PUTRESCINE ADDITION (RP 132)

Putrescine, mM	2,4-D, mg/L 0	0.01	0.1	0.5
	(20-Day Dry Weights, mg/mL)			
0	1.1	1.4	14.5	14.9
0.1	1.1	1.7	10.6	15.3
1.0	1.2	1.5	17.2	15.5

TABLE XV

10-D COT LAUNCH MATRIX (RP 140)

2,4-D, mg/L	LM3 Variations: Control	Sucrose↓	Ca↑ Mg↓	Zn↓ Co↑	Combo[a]	1/2 Strength
	(11-Day Fresh Weights, mg/mL)					
10 µL/mL I.D.						
0.5	38.7	49.6	27.1	52.3	36.2	39.3
0.1	30.8	34.1	25.4	35.9	23.2	23.5
0.01	14.2	22.1	6.0	8.6	16.7	15.0
0.00	10.1	14.1	10.7	11.6	7.7	11.1
3 µL/mL I.D.						
0.5	3.2	2.4	1.5	3.1	1.5	1.5
0.1	2.1	2.2	0.9	2.8	1.1	1.5
0.01	0.2	1.2	1.3	1.5	1.2	1.4
0.00	1.2	1.1	1.3	1.3	1.2	1.2

[a]Combo means sucrose↓ Ca↑ Mg↓ Zn↓ Co↑.

TABLE XVI

10-D COT LAUNCH MATRIX (RP 140)

2,4-D, mg/L	LM3 Variations					
	Control	Sucrose↓	Ca↑ Mg↓	Zn↓ Co↑	Combo[a]	1/2 Strength
	(11-Day Proanthocyanidin, mg/g fresh weight)					
10 µL/mL I.D.						
0.5	0.3	0.2	0.5	0.3	0.4	0.4
0.1	0.3	0.3	0.8	0.3	1.1	0.9
0.01	2.2	0.6	29.9	1.9	15.1	5.1
0.00	19.3	5.2	28.6	0.9	4.8	26.6
3 µL/mL I.D.						
0.5	2.6	2.1	2.5	2.4	5.4	12.5
0.1	4.1	3.2	2.0	4.7	4.1	15.9
0.01	6.0	3.2	2.7	8.2	17.1	29.8
0.00	12.4	3.4	9.8	19.2	12.2	23.5

[a]Combo means sucrose↓ Ca↑ Mg↓ Zn↓ Co↑.

TABLE XVII

LAUNCH MATRIX FOR 10-D COT AND FOUR NEW LINES (RP 142)

	Additives		
Auxin, mg/L	None	BAP, 0.1 mg/L	Putrescine Spermidine Spermine, 0.1 mM each
0	x	---	---
0.001 2,4-D	x	x	x
0.01 NAA	x	x	---

Triplicates on 3 basal media: (1) LM
(2) LM with 2% sucrose, Ca↑, Mg↓
(3) LM with 2% sucrose, Ca↑, Mg↓, Zn↓, Co↑

TABLE XVIII

10-D COT LIGHT LAUNCH PROTOCOL (RP 148)

Auxin, mg/L	
none	
0.5	2,4-D (control)
5.0	
1.0	NOAA
0.1	
5.0	
1.0	NAA
0.1	
0.01	

TABLE XIX

LAUNCH MATRIX FOR 10-D COT AND NEW CELL LINES (RP 198)

	1/2 LM Additives		
Growth Regulators, mg/L	None	0.1% (W/V) LP Pollen Extract	Agmatine 1 mM Putrescine 0.1 mM Spermidine 0.1 mM Spermine 0.1 mM
None	x	x	x
NOAA, 0.1 BAP, 1.0	x	x	x
NAA, 0.01 BAP, 0.1	x	x	x

Launch on 0.15% Gelrite in dark. > 63 µm.

Discussion and Conclusions

While there was little, if any, progress on the road to conifer somatic embryogensis in these launch experiments, it must be stressed that these experiments were not conducted with great expectations, since, except for polyamine additions, they were not based on our model system approach. In the

completely unmonitored cases, one is left essentially at the starting gate when there is no response. The modest amount of data on growth and tannin production collected from some experiments is discussed below as it may provide some basis for future directions.

In the 10-D Cot data of RP132 and RP140 it is quite apparent (Fig. 28 and 29) that there are problems of no growth and tannin production when one goes to less than "standard" (10 µL/mL) inoculum density. Note that even the 3 µL/mL inoculum is much greater than the 0.5 µL/mL used in the wild carrot launch experiments. With stock (parental) culture dilutions and >10 µL/mL inoculum densities there are neither growth nor tannin problems, most likely due to 2,4-D "carryover." The addition of putrescine in RP132 appeared to worsen the tannin production at intermediate levels of 2,4-D with little effect on growth, although it may be worth noting that the poorest growth at 0.1 ppm 2,4-D is associated with the putrescine concentration (0.1 mM) yielding the greatest tannin level. The same observation can be made for 1.0 ppm BAP at the 0.1 ppm 2,4-D level. Responses of 10-D Cot to the LM-3 variations in RP140 (Tables XV and XVI) is of some interest. Diminishing the sucrose concentration in LM-3 generally promotes growth and lowers tannin production as expected. This effect is grounded in lower C/N ratios as discussed in previous reports. The basis for designing the calcium/magnesium shift resides mostly in the fact that normal LM and LM-3 have very low calcium levels with respect to other common media. The results in Tables XV and XVI show that returning 10-D Cot to high calcium levels is not desirable in terms of growth (at high levels of 2,4-D) nor in terms of tannin production (at low levels of 2,4-D). The simultaneous reduction of zinc and elevation of cobalt produced positive growth effects (at high levels of 2,4-D) and a suppression of tannin production (at low levels of 2,4-D). We have

observed previously that cobalt suppresses cell browning presumably related to tannin production. However, in these data one does see an instance of strong suppression of tannin production (19.3 to 0.9) that results in little, if any, growth response (10.1 to 11.6) (cf. Discussion under Tannin Content and PAL Levels...).

The effects on tannin production of switching 10-D Cot from 2,4-D to NOAA or NAA (Fig. 32) suggest that one may be able to dilute these auxins to lower concentrations than 2,4-D without experiencing tannin accumulation. It should not be concluded on the basis of this data that NAA is better than NOAA in this respect, since no data were taken for NOAA at the 0.01 mg/L level.

Polyamines and a polyamine precursor (agmatine) were present in a number of these trials but were ineffective. This lack of response should not be upsetting for several reasons, but particularly because there is no reason to believe that polyamines alone could set 10-D Cot on the road to somatic embryogenesis. Neither is the lack of response to the pollen extract disturbing. Only one concentration was tried, and more emphasis has been placed upon immature seed extracts as discussed elsewhere in this report (see Influence of Natural Pine Cone Extracts on Wild Carrot Embryogenesis).

The failure to observe organizational effects in this series of experiments gives greater impetus to the model system approach. The latter may be slower, but it is likely to be a surer approach. Nevertheless, unmonitored or minimally monitored launches will continue to be scheduled, but in the future one can expect to see more parameters derived from model system research incorporated into these launch matrices.

CO-CULTURE OF WILD CARROT AND LOBLOLLY PINE IN LM

Introduction

In older literature concerning wild carrot somatic embryogenesis, there is some suggestion that the wild carrot cells "condition" the medium that they are growing in. The conditioning of the medium supposedly helps or allows them to develop into somatic embryos.

Because LM can support growth of wild carrot and gymnosperm cells, a study was undertaken to see if co-culturing the two species together might help the gymnosperm cells in their development.

Methods

Equal cell numbers of a competent wild carrot line (growing in LM + 0.5 ppm 2,4-D) and 10-D Cot (a loblolly line growing in LM + 0.5 ppm 2,4-D) were placed into duplicate flasks containing LM + 0.5 ppm 2,4-D. Approximately every 10 days the culture was subcultured into new flasks and fresh medium (with growth regulator). At the time of subculture, an aliquot of cells was taken to determine the number of cells of each species in the cell population. This was accomplished by rendering the aliquot of the cells into protoplasts and then counting the protoplasts based on the size differences between wild carrot protoplasts (10-15 μm diameter) and loblolly pine protoplasts (25-30 μm diameter, see Fig. 33). Viability of the protoplasts was checked by staining with Evans Blue. After 3 to 5 subcultures, aliquots of the mixed population were put through the normal wild carrot protocol for somatic embryo induction, except that cells were placed not only into liquid but also on agar. After 14-21 days the structures were examined to determine the species.

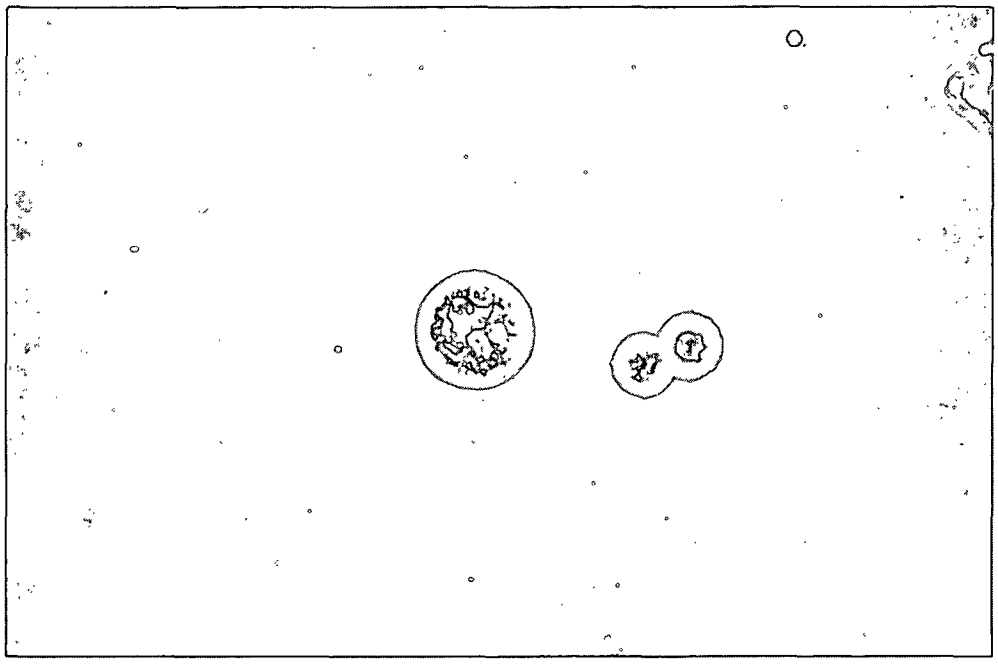


Figure 33. Light microscopy photograph of loblolly pine protoplast (large protoplast) and two wild carrot protoplasts (small protoplasts). 100X.

Results

Table XX shows how the population of suspension cells changed as the two species were grown together.

TABLE XX

PROTOPLAST POPULATION RATIOS FOR CO-CULTURES GROWN IN LM

Subculture	Ratio Wild Carrot/Loblolly Pine
0	1.14:1
1	1.06:1
2	14.5:1
3	7.4:1
4	5.6:1

Discussion and Conclusions

At the time of the initial subculturing, there was approximately a 1:1 ratio of wild carrot to 10-D Cot cells. This ratio maintained itself for the first subculture, but at the end of the second subculture the wild carrot cells seemed to have gained an advantage; the ratio had changed to 14.5:1. However, after the third and fourth subcultures, the population of wild carrot and loblolly pine cells seemed to stabilize to around 5 or 6:1.

After the first subculture the size differences between the wild carrot and loblolly pine cells became less distinct, (Fig. 34) and as a result were difficult to categorize. Thus counting the protoplasts based entirely on size was somewhat inaccurate and may need to be reevaluated. However, the most important aspect of this study was that when the mixed population of cells was launched there were no gymnosperm embryos produced (the wild carrot cells remained competent and produced large numbers of embryos).

Thus, the conclusions from this study are (1) wild carrot and loblolly pine cells can be grown together, and (2) co-culturing did not inhibit embryogenesis in the wild carrot nor did it stimulate development in the loblolly pine cells.

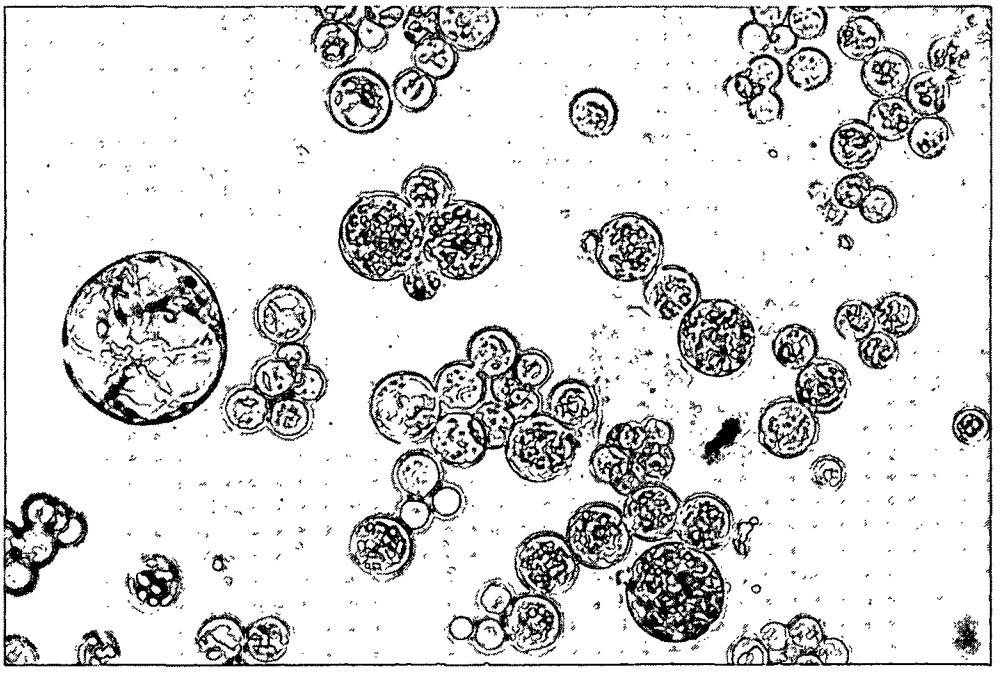


Figure 34. Light microscopy picture of mixed population protoplasts after third subculture.

PLANS

Research plans, if they are to be realistic, must be based on the current rate of progress, anticipated results of studies presently underway, breakthroughs of other researchers, funding, and manpower. When you carefully consider the several factors involved, plans at best represent statements on the general direction and the areas of emphasis of a research program. In the Institute's program, as more progress is made in defining the critical factors involved in natural and somatic embryogenesis, model systems research will be reduced. In the coming year, considerable emphasis is expected to be placed on generating new cell lines (Objective I) and running Objective II studies aimed at producing somatic embryos. Additionally, a small amount of our research effort is planned in the areas of plantlet transfer (Objective III)* and fidelity investigations (Objective IV)[1].

Model Systems Research

A considerable amount of model systems research remains to be completed. This is research that is aimed at establishing morphological and biochemical markers that the "team" feels are required to identify embryogenic cell lines. Of particular interest are markers that can be used during cell line buildup and in the early stages of launch experiments. Included under this phase of research are studies on:

(1) Polyamine levels and associated enzyme activity during the early stages of natural pine embryogenesis

(2) Changes in growth regulator levels (including ascorbic acid and glutathione) during natural conifer and wild carrot somatic embryogenesis

[1]For further information, the reader is referred to the overall research plan for this project that is presented on the last page of the Appendix.

(3) Coffee embryogenesis

(4) Growth rate and cell division during early stages of embryogenesis

(5) The influence of a number of alternative growth regulators on wild carrot somatic embryogenesis

(6) Factors important in the conversion of arginine to polyamines

(7) Energy (ATP) levels in wild carrot and loblolly pine natural and tissue culture systems

OBJECTIVE I RESEARCH

Research on generating and maintaining competent cell lines is expected to include:

(1) Generation of new cell lines from immature embryos, protoplasts, and microsporophyll tissue

(2) Determination of the influence of nitrogen sources, polyamines, gibberellins, and cytokinins on conifer cell line quality

(3) Determination of the influence of natural conifer extracts on conifer cell line quality

(4) Examination of the importance of light on cell line quality, as defined by a number of our standard biochemical markers.

OBJECTIVE II RESEARCH

Conifer somatic embryogenesis studies are expected to become an increasingly important part of our program during the coming year (84/85). Plans in this phase of the program are expected to include:

(1) Running monitored launch experiments with established cell lines that have as objectives the correction of apparent deficiencies and the reduction of inhibitors

(2) Conducting occasional unmonitored launch experiments incorporating a combination of promising factors

(3) Establishing monitored launch experiments using promising new cell lines

(4) Conducting studies with the objective of determining the influence of natural extracts on conifer embryogenesis

(5) Conducting studies on new growth regulators and stress as methods of triggering embryogenesis.

ACKNOWLEDGMENTS

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The research team also wants to express our appreciation to Kirk Milinek and associates of Weyerhaeuser Company and John Pait of Container Corporation of America, for their assistance in making collections of immature loblolly pine cones for biochemical studies and for use in the initiation of new cell lines.

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GLOSSARY

Adventitious - Roots, shoots, embryos, or other organs or tissues developing in an abnormal position.

Amyloplast - A colorless plastid modified for starch storage.

Archegonium - The flask-shaped container of the ovum (egg cell) of some gymnosperms. The swollen base (venter) contains the egg cell and is surrounded by the neck, with neck canal cells.

Aseptic culture - Surface sterilization of parental explants, free from pathogens, but not necessarily free of internal symbionts.

Asexual reproduction - Reproduction without fertilization. New individuals may develop from vegetative parts such as tubers, bulbs, or rooted stems, or from sexual parts such as unfertilized eggs or other cells in the ovule.

Auxins - A class of plant growth hormones of diverse makeup which cause cell enlargement, apical dominance, and root initiation.

Callus culture - Proliferation from a parental explant of many cells in protoplasmic continuity, but having no equivalence with any normal tissue. Same as tissue culture.

Cell differentiation - Internal chemical or ultrastructural changes preceding or accompanying specialization of function.

Cell suspension - Culture of single cells in moving liquid medium, often used to describe suspension cultures of cells and cell aggregates.

Chloroplast - A membrane-enclosed subcellular organelle containing chlorophyll. Chloroplasts are the sites of photosynthesis. They contain DNA and polysomes and can replicate.

Clonal propagation - Propagation of a group of plants derived from a single individual (ortet) by asexual reproduction. All members (ramets) of a clone have the same genotype and consequently tend to be uniform.

Coumarins - A class of phenylpropanoid phenolic compounds of which coumarin itself typifies the structures.

Cotyledon - The leaf formed directly from the embryo of an angiosperm or gymnosperm. There may be one (in monocotyledons), two (in dicotyledons), or several (in gymnosperms). They act as storage organs in nonendospermous seeds and as the first photosynthetic organs in endospermous seeds.

Cybrid - A hybrid cell resulting from the fusion of protoplasts from genetically distinct plants.

Cytokinins - A class of plant growth hormones associated with cell division, assisting with the transmission of the genetic information from the genes to the proteins.

2D TLC - Two-dimensional thin-layer chromatography.

Diploid - Having two sets of chromosomes in the nucleus. One-half of the chromosomes are contributed by one parent, one-half by the other parent. Many higher organisms are diploid except for their sex cells and associated tissue.

EM - Electron microscope.

Embryo - The young plant developing in the megagametophyte from the fertilization of an egg cell, or without fertilization. In aseptic cultures, adventitious embryos show polarization followed by the growth of a shoot from one end and a root from the other end.

Embryogenesis - Initiation of embryoids or embryos from cultured cells.

Embryoid - A cell group approximating an embryo, but having a more random cell arrangement.

Empirical method - Method based solely on experiment and observation.

Enzyme - A protein molecule that catalyzes a specific chemical reaction.

ER - Endoplasmic reticulum. A system of membranes (originating from the external membrane of the nuclear envelope) that permeates the cytoplasm and that may or may not be covered with ribosomes.

Erosion zone - Zone in the gametophytic tissue below the archegonium that is degraded by the developing embryo.

Eucaryotic cells - Cells with true nuclei bounded by nuclear envelopes and which undergo meiosis.

Excise - To cut or isolate callus tissue from its parental explant or to remove adventitious shoots from callus tissue for rooting.

Explant - A plant part excised and prepared for aseptic culture by surface sterilization followed by the exposure of live cells to a nutrient medium.

Fertilization - The normal union of two gametes during sexual reproduction.

Flavonoids - A class of phenolic compounds usually consisting of two hydroxylated aromatic rings joined by a three-carbon chain.

Gametophytic tissue - Haploid tissue of the seed that surrounds the developing embryo during the latter stages of embryogenesis.

Gene - One of the units of inherited material carried on a chromosome; arranged in a linear fashion and indivisible.

Gene pool - Reservoir of genetic variability available for use in genetic improvement of tree species.

Genetic gains - Average improvement in progeny over the mean of the parents.

Genetic variability - The variation existing in a given population (species, for example) with respect to particular genes or arrangement of genes.

Genotype - The genetic makeup of an individual; carried in the chromosomes.

Groundplasm - Homogeneous plasma (matrix) remaining after cell organelles and particles have been excluded.

Haploid - Having the reduced chromosome number, i.e., having one set of chromosomes in the nucleus. This is normal in sex cells, which have only half the number of sets occurring in diploid vegetative cells.

Hormone - Any growth substance which is generally transported to the site of action and can stimulate growth or cell enlargement (auxins), cell division (cytokinins), stem elongation (gibberellins), or can retard growth as in the abscission of leaves (ethylene).

Hybrid vigor - The increase in vigor, size and fertility of a hybrid as compared with its parents, resulting from the union of genetically different gametes and assumed to be due to special recombinations of dominant and recessive genes (heterosis).

Hybridization - The production of offspring of genetically different parents.

Hypocotyl - The part of a seedling axis between the radicle and the cotyledon(s).

Induction - To cause initiation of a plant structure, organ or process.

Inoculum - A small piece of tissue cut from callus, or a small amount of cell material from a suspension culture placed in contact with fresh medium for continued growth of the culture. Inocula (plural).

Interspecific hybrid - The progeny from matings between species.

Intraspecific hybrid - The progeny from matings within species.

In vitro - Outside the living organism.

In vivo - Within the living organism.

Isozymes - Multiple forms of a single enzyme.

Lipids - Any of a group of biochemicals which are variably soluble in organic solvents and barely soluble in water.

Milieu - The whole chemical and physical environment of a culture.

Meristem - A localized group of cells, actively dividing and undifferentiated but ultimately giving rise to permanent tissue such as shoots, roots, wood or bark.

Meristemoid - A localized group of cells in callus tissue, characterized by an accumulation of starch, RNA and protein, and giving rise to adventitious shoots or roots.

Mitochondria - Small bodies in spaces of the ground cytoplasm. They are spherical, long rods, or threads, and are the sites of many important enzymatic processes. The inner layer of the wall is infolded into fingerlike processes.

Morphogenesis - Initiation of organized tissue in callus or suspension cultures.

Nonrelated species - Species that are members of different genera.

Nutrient medium - A solid or liquid combination of major and minor salts, an energy source (sucrose), vitamins, hormones, and occasionally other defined or undefined supplements. Usually made up from previously prepared stock solution, then sterilized by autoclaving or filtering through a micropore filter. Media (plural).

Organized tissue - Tissue composed of regularly differentiated cells.

Organelle - A complex cytoplasmic structure of characteristic morphology and function, such as a mitochondrion or plastid.

Organogenesis - Initiation of roots or shoots from callus meristemoids.

Parasexual hybridization - Hybridization resulting from asexual fusion of cells, either diploid or haploid.

Passage - The duration of growth of callus or cell material from one subculture to another.

Plasmalemma - The semipermeable unit membrane surrounding and containing the cell cytoplasm. In plant cells, it is pressed up against the inner surface of the cell wall.

Polyploidy - Having three or more times the haploid number of chromosomes.

Procaryotic cells - Single-celled organisms and reproducing entities that lack a membrane-bound nucleus; they do not undergo meiosis; these include the viruses, bacteria, and blue-green algae.

Protoplast - Spherical cell protoplasm (cytoplasm + nucleus) bounded by a membrane but no cell wall.

Protoplast fusion - Union of two protoplasts into one cell.

Ribosomes - Organelles containing protein and RNA. They are seen as dense particles in electron micrographs. They are found in all types of cells in which protein is being synthesized.

SEM - Scanning electron microscope.

Somatic - Diploid body cells of an organism; those cells other than germ cells.

Somatic cell hybrid - The plant resulting from fusion of protoplasts from somatic cells of genetically different sources.

Subculture - Cutting solid callus into small cubes (inocula) for transfer to fresh medium. Sometimes used to denote the adding of fresh liquid medium to a suspension culture.

Suspension culture - Cells or cell aggregates dispersed and growing in moving liquid medium.

Tannins - A class of complex phenolic compounds known for their astringency and ability to tan the proteins of animal skins. There are two major types of tannins, the hydrolyzable and the condensed tannins.

TEM - Transmission electron microscope.

Tissue culture - General term for callus and cell cultures.

Totipotency - A cell characteristic in which the cell retains the potential of forming all the cell types of the adult organism.

Ultrastructural - Sublight microscopic, intracellular structure.

Vacuole - A fluid-filled space in a cell. A single vacuole, taking up most of the volume of the cell, present in many plant cells, and containing a cell sap which is isotonic with the protoplasm.

Vegetative cells - Nonreproductive cells such as haploid cells from female gametophytes of conifers or diploid somatic cells.

Vesicle - Small membrane-bound body in the cytoplasm.

Zygote - Fusion product of male and female sex cells or fusion product of protoplasts.

TABLE XXII

AMINO ACIDS ABBREVIATIONS

ala	alanine
arg	arginine
asn	asparagine
asp	aspartic acid
cit	citrulline
cys	cysteine
γ-aba	aminobutyric acid
gln	glutamine
glu	glutamic acid
gly	glycine
his	histidine
hyp	hydroxyproline
ile	isoleucine
leu	leucine
lys	lysine
met	methionine
orn	ornithine
phe	phenylalanine
pro	proline
ser	serine
thr	threonine
trp	tryptophan
tyr	tyrosine
val	valine

TABLE XXIII

CUMULATIVE LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ADC	Arginine decarboxylase
ADP	Adenosine diphosphate
AOA	Aminooxyacetic acid
AOPP	α-Aminooxy-β-phenylpropionic acid
ATP	Adenosine triphosphate
BAP	Benzylaminopurine
cAMP	3',5'-Cyclic adenosine monophosphate
C/N	Carbon/nitrogen
DF	Douglas-fir
DFMA	α-difluoromethylarginine
DFMO	α-difluoromethylornithine
DCHA	Dicyclohexylammonium sulfate
FAA	Free amino acid(s)
G-1-P	Glucose-1-phosphate
GA	Gibberellic acid (gibberellin)
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
HFBI	Heptafluorobutyrylimidazole
HPLC	High performance liquid chromatography
IAA	Indoleacetic acid
IBA	Indolebutyric acid
IPA	Isopentenylaminopurine
LM	Litvay medium
LP	Loblolly pine
lx	Lux
MEOI	Methyleneoxindole
MES	Morpholinoethane sulfonic acid
MOI	Methyloxindole
MOPS	Morpholinopropane sulfonic acid
MGBG	Methylglyoxal bis-guanyl hydrazone
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NBT	Nitrobluetetrazolium
NOAA	Naphthoxyacetic acid

TABLE XXIII (Continued)

CUMULATIVE LIST OF ABBREVIATIONS

OBHA	o-benzylhydroxylamine
ODC	Ornithine decarboxylase
P	Putrescine or phosphate
PAL	Phenylalanine ammonia lyase
PPi	Pyrophosphate
ProA	Proanthocyanidin
RP	Red pine or research plan
Sd	Spermidine
SIM	Selection ion monitoring
Sp	Spermine
TLC	Thin-layer chromatography
TrpAM	Tryptamine
UDP	Uridine diphosphate
UDPG	Uridine diphosphate glucose
UTP	Uridine triphosphate
WC	Wild carrot
WCM	Wild carrot medium
WP	White pine

APPENDIX

TABLE XXIV

ANALYSIS METHODS FOR PHOSPHORUS
Fiske & Subbarow, 1925

Standard: KH_2PO_4 - 10 mM, M. W. = 136.1

Reagents:

A. 4 g Sodium molybdate in 96 mL R.O. H_2O
B. 10N H_2SO_4 = 26.8 mL conc. H_2SO_4 to 100 mL R.O. H_2O

Mix A & B 1:1 by volume

C. Developer - 1 g Elon (photo developer), 3 g $NaHSO_3$, 96 mL R.O. H_2O

Procedure:

Pipet into small test tube - 0.3 mL A & B mixture, 0.3 mL sample, 2.1 mL H_2O, 0.3 mL of C (developer). Mix and let stand 15 min. Read absorbance against water at 25°C at 660 nm within one hour.

TABLE XXV

FLOW DIAGRAM FOR IAA EXTRACTION METHOD

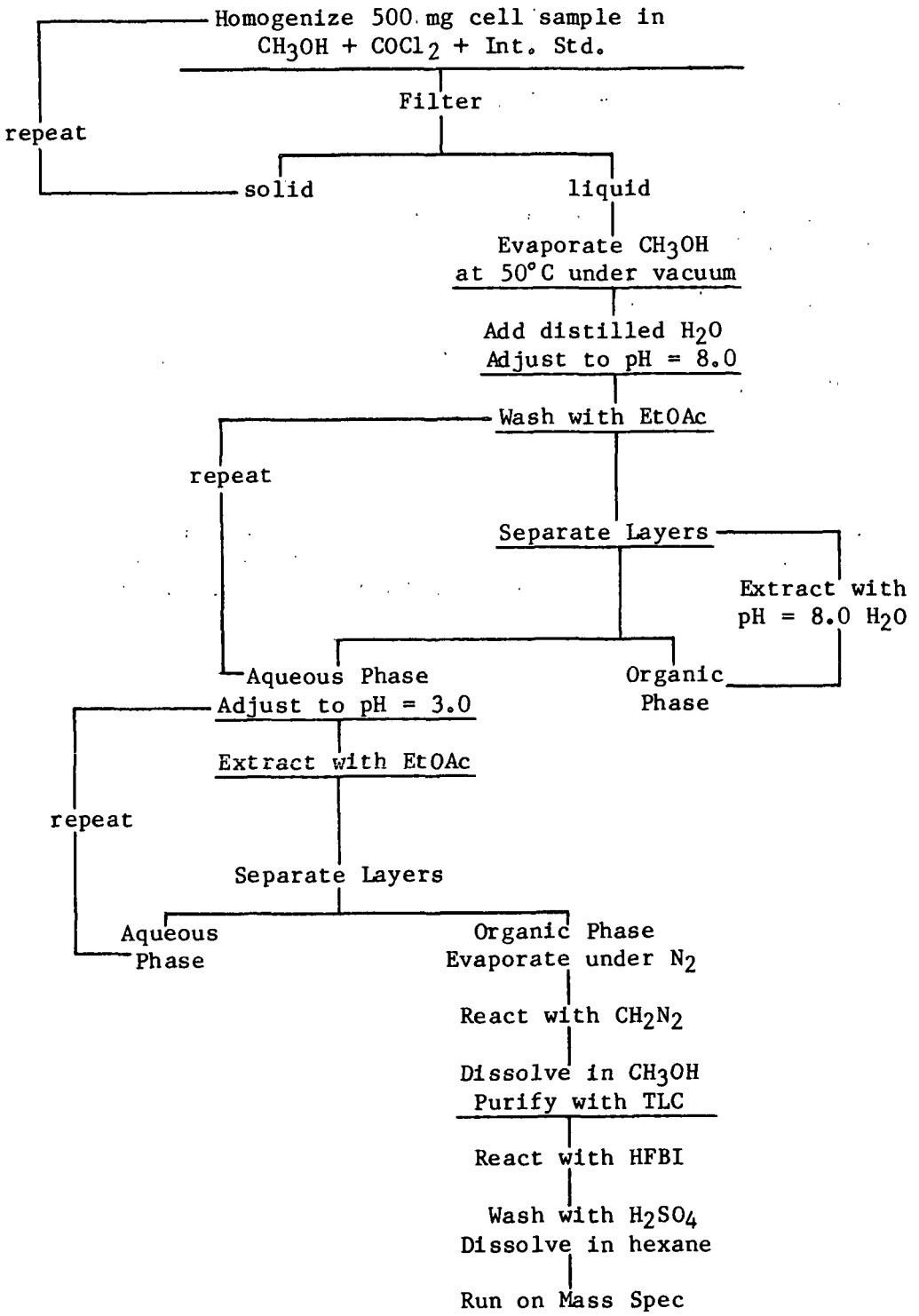

TABLE XXVI

CONIFER TISSUE CULTURE OBJECTIVES